

Biodegradable Thermogels

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Biodegradable Thermogels

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Preface

How did I get myself into the field of thermogels? That is a combination of various stories and chapters in my scientific life. My initial interest in thermogels stemmed from my pre-undergraduate days in 1997. I read a paper published in *Nature* by the now Professor Byeongmoon Jeong. It was his PhD work at that time (Biodegradable block copolymers as injectable drug-delivery systems, *Nature*, 1997, **388**, 860). Something about that work really fascinated me; it might have been the temperature responsiveness or the potential of creating an injectable drug delivery depot or just because it was such an elegant new material then. I thought about how I could use that for various applications. However, due to limited synthetic experience, I could not make these materials on my own. So the fascination had to take a break while I picked up basic chemistry skills in the university. I never forgot that paper and got to work on the topic during my PhD in 2007. That, in itself, is also a story.

My laboratory then was working on the direction of developing new cyclodextrin host guest materials, which was a nice academic endeavor but really a crowded area of research. I needed something more exciting and took to developing a new type of thermogel in the laboratory. As this was a direction that was different from the laboratory's focus, I had total freedom on what I could develop. *The catch?* I had no funds and my supervisor mentioned (half in jest, I hoped) that I could look into the chemical inventory and come up with whatever from what was available. I took up the challenge and developed the thermogel that demonstrated the lowest critical gelation concentration at that time and is still the current lowest ever reported. It was a really proud moment as I had conceptualized, designed and synthesized the material entirely from scratch through my own wits and effort. I worked hard and towards a quick graduation in 2009 within 2 years of my candidature and embarked on my independent scientific career.

Today, in 2018, as I sit here reflecting, I feel that many things have been learnt about these thermogels and that I am very grateful to be given this opportunity to curate all of this information in the form of a book. This book is a culmination of more than a year of preparation and hard work by everyone involved. As the two co-editors of this book, David and myself would like to acknowledge the immense effort put in by all the authors. We would also like to acknowledge the guidance and patience of the Royal Society of Chemistry team, particularly Michelle Carey and Connor Sheppard. I would also like to take the time to acknowledge the various key people for their support and collaboration throughout the years. My first acknowledgement goes to Professor Yunlong Wu, from my ex-PhD laboratory, for sticking by me all these years and working closely together to test the *in vivo* efficacies of these thermogels. Next acknowledgement goes to Dr Zibiao Li, also from my ex-PhD laboratory, for being the nucleus of my research team in IMRE, A*STAR. He has taken on a lot of the directing of the research work in my group as I undertook more and more administrative duties. I am also grateful to my clinical collaborators, Professor Gopal Lingam, Drs Xinyi Su and Zengping Liu. We are pushing really hard to get these thermogels into clinical trials, so please watch this space. I would like to thank my staff, Drs Sing Shy Liow and Lu Jiang, for doing most of the synthesis now that I am quite useless in the laboratory. Finally and most importantly, I am extremely grateful to my parents, Moi Joo Loh and Joo Gek Lim for always being there for me, for every dinner time discussion of my work, for trying to understand the things that I wrote in my manuscripts and for encouraging and supporting me all the way. All my thermogel work is dedicated to my late grandfather, Seak Liang Lim, for being my role model, for sparking my creativity in various aspects of my life and for just being around for me and listening to a “really strange technology” (as he knew it) during my university and PhD days. He passed away in the year that I graduated with my PhD and I hope that I have made him proud with my achievements in this area since then.

Xian Jun Loh
David James Young

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CHAPTER 1

Thermogelling Polymers and Their History

OWH CALLY,^a DAVID JAMES YOUNG^{a,b} AND XIAN JUN LOH^{*a}

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1.1 Introduction

The evolution of the biomedical industry to cope with intricate medical problems has always relied on advances in biomaterials (Figure 1.1).¹ Primary among the properties required of these materials was bioinertness, meaning that they could perform largely mechanical functions well with minimal interaction with the host systems, thus minimizing the chance of biological rejection. However, later developments have seen the phasing out of inert and unresponsive materials, bringing about the era of bioactive materials that can interface with the physiological environment to elicit appropriate biological responses (Figure 1.2).

As biomaterials ascend in increasing sophistication, they have become more responsive to external stimuli. This has resulted in a new class of “smart” biomaterials: biomaterials that respond to changes in environmental pH, temperature, light and more.^{2,3} These materials, also known as stimuli-responsive, environmentally-sensitive or intelligent materials, exhibit an observable change in their properties upon the stimulus.

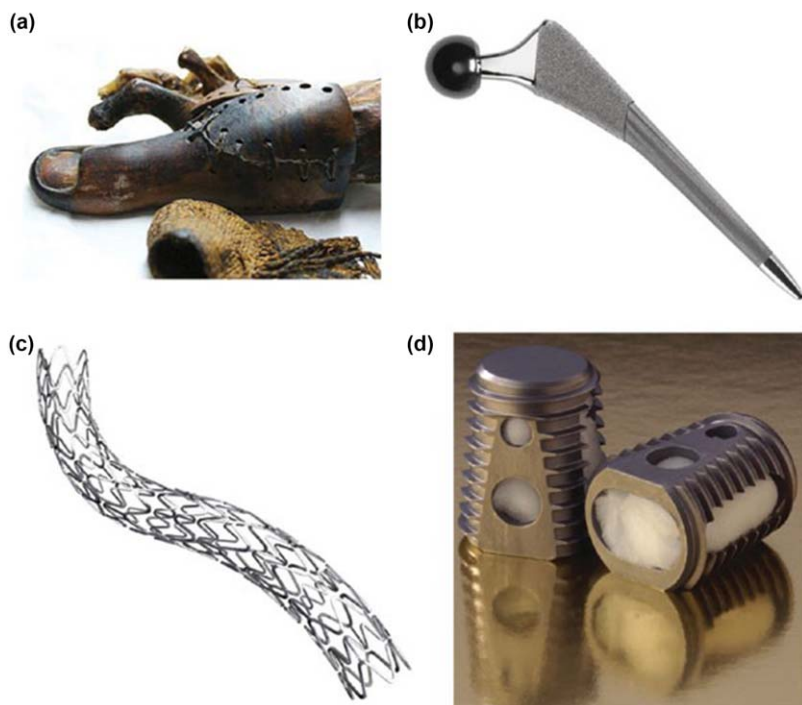


Figure 1.1 Images of biomedical devices fashioned from various biomaterials, from traditional prosthetics to new state-of-the-art devices. Reprinted by permission from Macmillan Publishers Ltd: Nature (ref. 1), copyright (2009).

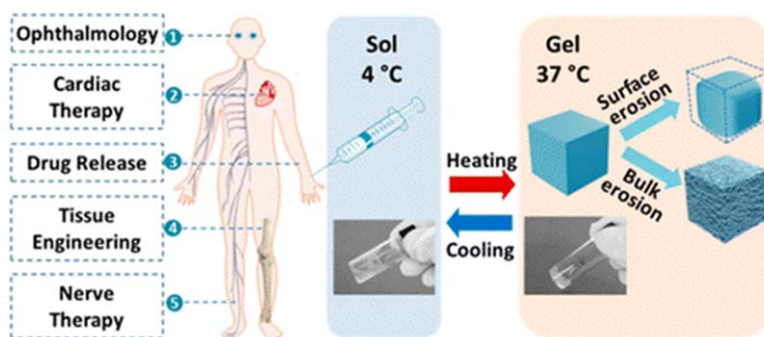


Figure 1.2 Thermogelling materials and the applications that can be derived from them. Reprinted with permission from ref. 7. Copyright 2016 American Chemical Society.

Such changes include modification of their shape, solubility, surface characteristics and the ability to self-assemble or undergo sol-to-gel phase transition (Figure 1.2).³

Smart thermogelling materials possess both high water content and the tunable properties of hydrogels, with the ability to respond to external temperature changes with a simple, physical and reversible sol-to-gel phase transition.^{4–6} Thermogels have been proposed for many uses including drug delivery, gene delivery and scaffolding for tissue engineering.^{7–11} The thermogelling Pluronic systems have been an important contributor in the biomedical arena since their introduction about 70 years ago. Also known by their non-proprietary name, poloxamers, Pluronics were first brought onto the 1950s commercial scene by BASF, and have now gained increased industrial footing, being sold by various companies under a variety of other tradenames, such as Kolliphor. First introduced as surface active agents (surfactants), Pluronics, which were under the category of non-ionic triblock copolymers, are characterized by a central hydrophobic propylene oxide block in between hydrophilic ethylene oxide blocks. By arranging the water-soluble hydrophilic blocks and water-insoluble hydrophobic blocks, the molecular weights and the ratio of the weight of the hydrophilic to hydrophobic blocks can be tuned, allowing for the synthesis of a family of surfactants with systematically varying physical properties and similar chemical properties to ether alcohols. This thus allowed for their optimization in a variety of different biomedical applications.¹²

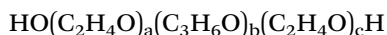
Compared with other well-known surfactants, such as ethoxylated fatty alcohols or alkyl phenols, Pluronics can be differentiated by four characteristics. Firstly, Pluronics have much higher molecular weights than other surfactants, reaching a range of 1000 to 15 000. Secondly, their triblock structure possesses two hydrophiles, unlike the single hydrophile found in most non-ionic surfactants. The existence of ether oxygen atoms in both their hydrophobic and hydrophilic blocks also allowed Pluronics to form hydrogen bonds in their hydrophobic segments, which is not a feature of ethoxylated fatty alcohols or alkyl phenols. Finally, these copolymers exhibit a micellar arrangement in aqueous solution that is different from the hydrated spherical formations observed in other non-ionic surfactants.¹²

These unique structural, physical and thermos-responsive properties of thermogelling Pluronics have allowed them to remain very relevant in the biomedical sector decades after their introduction. In this chapter, we aim to examine the Pluronics systems in greater depth and detail their evolution as biomaterials.

1.2 Synthesis

The synthetic procedure for making Pluronic copolymers has been detailed by their inventor, Irving Schmolka. It first involves the formation of a polyoxypropylene glycol hydrophobe (minimum molecular weight of 900), by adding propylene oxide to a propylene glycol initiator in the presence of an alkaline catalyst. This must be conducted in an inert and anhydrous environment, under elevated pressure and temperature. Following the complete reaction of all the propylene oxide, two polyoxyethylene blocks, which

serve as the hydrophilic segments, are formed through the controlled addition of ethylene oxide. The mixture is then neutralized by adding phosphoric acid or other inorganic or organic acids.¹³ The above process results in the characteristic structure of the Pluronic copolymer which can be represented as:¹³



Where a is statistically equal to c , and adds up to 10–90% of the total polymeric weight, and b is at least 15.¹²

1.3 Micellization and Thermogelling Properties

1.3.1 Gelation Mechanism

Within the range of possible thermoresponsive properties, one of the most highly researched examples is gelation behaviour. This is due to the fact that thermoresponsive gelation brings with it an entire host of potential applications, including minimally invasive *in situ* formation of gels, or triggered release of actives (Figure 1.3).

During gelation, a solution of macromolecules or particles that exists in a liquid phase as a flowing fluid can be converted into a solid with elastic properties that can maintain its structural integrity and remain non-flowing throughout the duration of the experimental time scale.^{14,15} Conventional gelation behaviour can be observed in many systems, such as gelatin solutions, which gel upon being cooled down to a certain temperature. Typically, gelation occurs through the random increase of the number of either physical or chemical bonds that exist between the particles or molecules, which eventually results in the formation of a continuous connected network. In the case of physical bonds such as hydrogen bonds or Van der Waals forces, the gels formed can be re-converted back into their liquid state through modifications to the physical environment, such as through a change in temperature or pH. By contrast, chemical bonds are covalent in nature, and tend to result in permanent, non-reversible cross-linkage.¹⁴

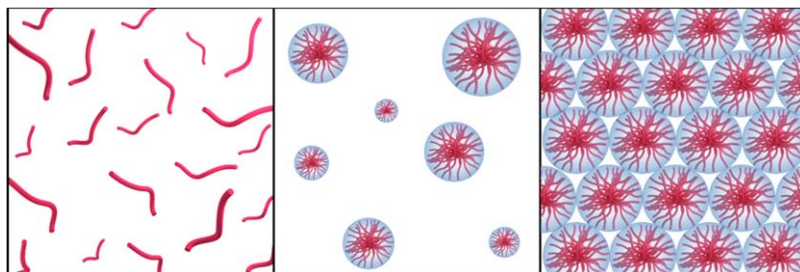


Figure 1.3 Illustration depicting the critical micelle concentration and critical gel concentration for block copolymers in solutions.

The aqueous gelatin solution has been a commonly explored example that exhibits thermoreversible gelation behaviour. It is able to undergo this process at concentrations above 2–3% (w/w), at a gelation temperature of approximately 30 °C. Thus, the typical procedure for the fabrication of gelatin gels involves preparing a hot gelation solution at around 50 °C and thereafter allowing it to cool to about 0 °C. At the start of the process, the solvated gelatin chains predominantly exist in random coil conformations of either α -type or β -type chains. However, the formation of the gel arises from the intertwining of the gelatin chains through the creation of hydrogen bonds to form a triple-helix structure. The dynamics involved are currently thought to consist of three discrete steps: the formation of aggregates from monomers, the disorder-to-order transition of random coils to single helices, and the order-to-order transition of the single helix to triple helix transition.¹⁶

So far we have described the gelation behavior of conventional gels. However, thermogels, the class of hydrogels to which the Pluronics series belongs, are thought to be a separate category from these. Although these copolymer systems can transition from a solution to a gel phase by virtue of the supramolecular interactions of their polymeric blocks, they differ from conventional gels, as this happens during temperature elevation. Thus, these systems exist as aqueous polymeric solutions in a non-viscous fluid phase when temperatures are low, and, upon an increase in temperature, such as to body temperature, are able to form a solid hydrogel. This phenomenon, at first impression, seems to contradict conventional sense, whereby the elevation material temperature should result in a solid-to-liquid transition due to melting. However, this conversion, which is thermoreversible, occurs *via* an entirely different mechanism of polymeric chain hydration and dehydration.¹⁷

To begin to look into this mechanism, the general properties of thermogelling copolymers must first be examined. Thermogelling copolymers are known to be amphiphilic macromolecules with a delicate balance of hydrophobic and hydrophilic properties of their different segments. This balance allows for the solvation of the copolymers in an aqueous solution during low temperatures *via* the occurrence of hydrogen bonding between water and the copolymers. These hydrogen bonds, however, are weakened upon the introduction of increased thermal activity through heating, which causes random motion of the molecules. This thus provides the chance for the random association of the copolymers' hydrophobic parts, which results in the formation of crosslink points in the form of nano-domains. Thus, at low concentrations with heated settings, the self-assembly of the copolymer chains results in their aggregation into micelles. This forms a corona of hydrophilic components and a core of hydrophobic components in the supramolecular structure. The above phenomenon, however, is not sufficient to induce gelation at low polymer concentration. An increase in polymer concentration brings about an increase in the number of micelles, which results in a denser packing within the solution. Only at the critical gelation

concentration (CGC) are the micelles packed closely enough to cause the formation of a gel state. Thus, this is why the sol-to-gel phase transition of a thermogel is both temperature and concentration dependent.¹⁷

The popularity of the Pluronics series can be directly attributed to its capability for exhibiting this thermogelling behaviour through self-assembly upon a temperature change. Below a specific temperature and/or concentration, known respectively as the critical micelle concentration and temperature (CMC and CMT), the copolymers exist as unimers, which are unaggregated individual coils in an aqueous solution. This phase, known as the sol phase, is characterized by fluidic flow. The increase in temperature of the solution and/or copolymeric concentration allows for the formation of thermodynamically stable micelles. However, the mechanism of gelation in Pluronics systems has been a point of contention between researchers over the years. Conflicting results observed using ultracentrifugation or light-scattering techniques indicated the lack of micellization in Pluronic solutions, while dye-solubilization and surface tension techniques could effectively determine CMC values.¹⁸ Later studies featuring static-light-scattering techniques also reported a lack of aggregation, which was contradicted by micellization associations detected through photon correlation spectroscopy and viscosity measurements. It was in 1987 that a study suggested three different regions of the micellization process: a unimer region, an equilibrium region consisting of a mixture of unimers and micelles, and a micelle region, suggesting that early controversies were due to observations made in only one of the three regions.¹⁹ However, among the studies that managed to observe micellization, different gelation mechanisms were proposed by different groups. Studies reported in 1983 observed a decrease in the CMC with increasing temperature. This led the researchers to conclude that the gelation could be attributed to an intrinsic change in the properties of the micelle, such as in their aggregation number or symmetry. However, a year later, a similar study observed a change in the ¹³C nuclear magnetic resonance chemical shift and peak broadening of the poly(phenylene oxide) (PPO) methyl group at the transition temperature, concluding that this was the result of dehydration of PPO from the existing micelles, which caused increased amounts of friction between the chains of copolymers, resulting in an increased solution viscosity and formation of a gel phase.¹⁵ In the same year, further studies resulted in the proposition of an entropically driven gelation mechanism involving the increase in overall disorder upon the squeezing out of ordered water molecules caused by interactions between the hydrophobic segments of the copolymers.¹⁹ It was also later proposed that the gelation was driven by the dehydration of the polyethylene oxide segments upon temperature increase.¹⁵

Many varying theories culminated in a paper in 1995 that proposed that the gelation was driven by the reduced polarity of ethylene oxide and propylene oxide segments upon temperature increase, as well as the entropically favourable hydrophobic effect, which is described as a gain in entropy in the surrounding water upon the aggregation of the unimers.²⁰ A recent review

thus proposed the following system. At a low temperature above the CMC, the copolymers begin to form micelles until they reach equilibrium with the unimers. Further temperature increase sees an increase in the volume fraction of the micelles due to the equilibrium shift towards the micelles, which correspondingly results in the reduction of unaggregated unimers. When the micelle volume fraction has increased beyond a certain limit (0.53), hard-sphere crystallization, or micelle packing, can occur and cause the system to become a gel.³

1.3.2 Kinetics of Micellization

The kinetics behind block copolymer micelles were thought to be different from surfactant micelles and observed through experiments that looked at the dynamics at equilibrium and dynamics of micellization. From these experiments, dynamics at equilibrium were attributed to an insertion-expulsion mechanism based on randomization kinetics that were independent of the concentration of polymer. By contrast, the dynamics of micellization were similar to surfactant micelle kinetics and thought to exhibit two different processes, namely a fast and a slow process. The former process was associated with the insertion of free copolymers into existing micelles, resulting in the aggregation of metastable micelles, while the latter was associated with either insertion-expulsion or fusion-fragmentation.²¹

1.3.3 Formation of Micelles with Different Morphologies

As mentioned in the introduction, micelles associated with Pluronic systems have the ability to form shapes different from the conventionally observed spherical shapes. In particular, there have been observations of sphere to rod micelle transitions attributed to the random fusion and fragmentation of the micelles instead of an ordered successive addition of micelle spheres to rod-like micelles.²¹ This difference in aggregate morphology can affect their suitability for different applications and thus further expands the range of purposes Pluronic thermogels can serve. For example, the potential to form vesicular and spherical micelles is often associated with drug delivery matrices, whereas worm-like micelles are applicable to areas such as oil extraction (Figure 1.4).²²

1.4 Pluronic Systems in the Biomedical Sciences

1.4.1 Early Uses

Pluronics in early medical research manifested in a variety of different end applications. They were largely used for purposes related to issues with fat and blood, serving to prevent thrombolysis, diminish fat emboli and hemolysis during cardio-pulmonary bypasses, reducing platelet adhesiveness and blood viscosity and to emulsify fat.²³ While these purposes were

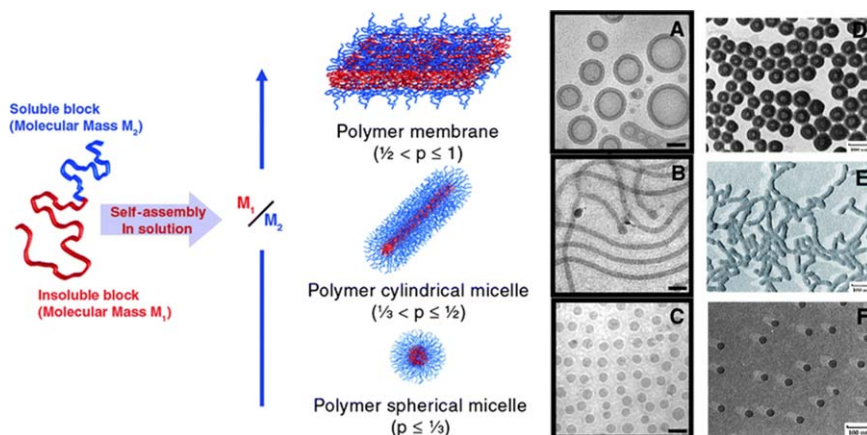


Figure 1.4 Polymeric micelles of various morphologies. Reproduced from ref. 48 with permission from The Royal Society of Chemistry.

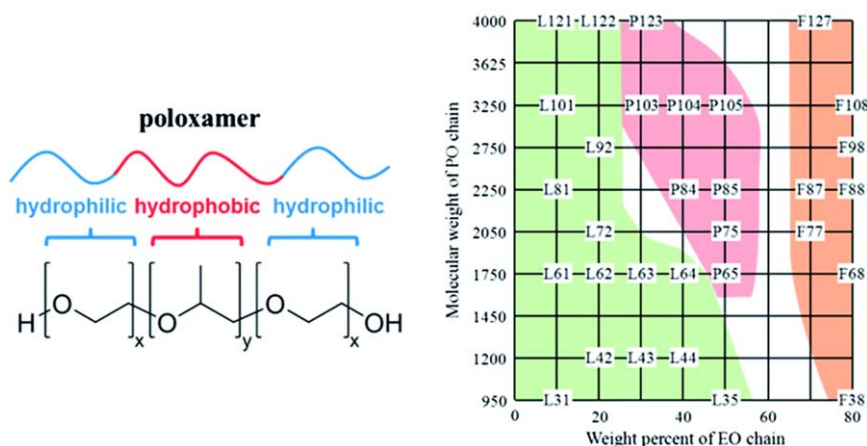


Figure 1.5 The gelation graphs of various Pluronic copolymers. Reproduced from ref. 23 with permission from The Royal Society of Chemistry.

limited to the copolymers themselves, and not in view of their capabilities as stimuli-responsive materials, they formed a foundation of research that established the Pluronics family as almost nontoxic materials (Figure 1.5).

1.4.2 Wound Healing

The first notable entry of Pluronic thermogelling systems into the bio-medical arena was proposed by their original inventor. In his paper, Irving Schmolka detailed the potential for Pluronic F-127 to be made into an

“artificial skin” suitable for the healing of burns by incorporating silver salts and other medicines into cold aqueous F-127 solutions before increasing the temperature and allowing the gels to form. These gels could be useful for topical application onto burnt or abraded skin. In determining their suitability for such purposes, Schmolka examined the toxicological properties of the Pluronics and concluded that they ranged from having very low toxicity at a very low molecular weight to being completely nontoxic at a higher molecular weight and concluded that there should be no hazardous effects if these systems were used for this application.²³

Several other studies have since explored the possibility of Pluronic systems as candidates for wound healing applications. The use of Pluronic F-127 in the standardized treatment of third-degree burns as a skin substitute was investigated. This study observed enhanced rates of healing at burn sites where the F-127, with propylene glycol added as a humectant, was applied as a reversible sol to gel “bandage” and investigated the potential for the addition of bacteriostatic or bactericidal agents to delay infection.²⁴

Later revelations with respect to wound healing indicated it to be a complex, localized process that involves many aspects, notably inflammation, wound cell mitosis and migration, and neovascularization, as well as the regeneration of the extracellular matrix. This led to an interest in improving wound healing systems beyond anti-infection, moving towards promoting wound healing. Studies thus began to look at Pluronic gel encapsulation of various growth factors that were thought to play significant roles in wound healing.²⁵ Encapsulation of growth factors such as epidermal growth factors and transforming growth factors were investigated in several studies in the 1990s, and pointed towards the growing prominence of Pluronic thermogels as topical growth factor delivery systems (Figure 1.6).^{25,26}

The beginning of the twenty-first century saw scientific research looking to fine-tune the properties of the Pluronic thermogels through mixtures of family members. Se *et al.* sought to develop a polymer solution that could exist as a gel coating on the injured surface to prevent postsurgical tissue adhesion. They combined Pluronic F-127 with Pluronic F-68. F-127 was notable for transitioning from a sol to gel state when heated to human physiological temperature, but was consequently difficult to handle at ambient temperatures because of this propensity for low-temperature gelling. On the other hand, F-68 only underwent transition at 40 °C and existed in sol state at physiological temperatures. Hence, the combination of the two at different ratios, together with mildly crosslinked alginate and ibuprofen resulted in Pluronic mixtures with controllable transition temperatures that could be applied in their liquid form to sites of injury, where they gelled and were stably maintained in that state.²⁷

The interest in Pluronic thermogels as wound healing systems has lasted well into current times, triggering the study of a Pluronic F-127 gel as a wound healer in itself. This work made use of the F-127 gel in normal saline solution without additional actives, and discovered that the mere topical application of these gels could enhance the healing of cutaneous wounds in

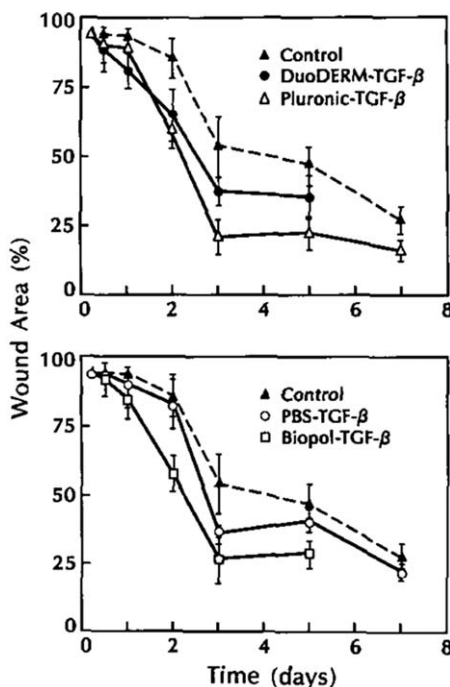


Figure 1.6 The sol-to-gel phase transition plots of Pluronic F-127/F-68 blends, indicating a variance in gelation temperatures when the copolymer ratios were varied.

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rats, significantly increasing the rate of wound closure. This was observed along with the increased expressions of vascular endothelial growth factor (VEGF) and transforming growth factor-beta 1 ($TGF-\beta_1$), growth factors which are thought to be key players in the wound healing process, serving to recruit inflammatory cells and ultimately aid in angiogenesis and granulation tissue formation. This thus led the authors to suggest that F-127 was mildly inflammatory in nature, and served to quicken wound healing by stimulating the expression of the aforementioned growth factors to take part during the inflammatory and proliferative stages of healing.²⁸

Although early research has already long advocated the use of Pluronic gels as candidates for wound healing, this was before the discovery that the systems in themselves could stimulate the quickening of the process. The systems were thus only thought of as advanced “bandages”, or vessels for the delivery of actives. However, the discovery of the inherent ability of Pluronic F-127 to aid in wound healing will lead to more investigations into the other members of the Pluronic family. This also brings about more reasons for the employment of the gels in such purposes, as their innate ability can be used synergistically with their other capabilities, such as encapsulation or preventing tissue adhesion, to bring about greater advances in this area.

1.4.3 Drug Delivery

The potential for micellization in Pluronic systems meant that they were capable of acting as encapsulants for drug delivery (Figure 1.4). The possibility of Pluronic systems incorporating other compounds had already been investigated early in their development when Schmolka successfully incorporated silver salts and other medicines into his gels. There is an overlap between drug delivery and wound healing methods, as the latter also seeks to deliver drugs and growth factors to accelerate the process of healing, and might hence be considered a subset of the former. The two areas, however, do diverge in several ways (Figure 1.7).

The introduction of medicine-holding Pluronic gel systems for burn healing sparked many investigations that followed largely similar paths. It also drew attention to the capabilities of these systems as drug carriers, beyond traditional dermal applications. Their micellar aggregations rendered them highly attractive as encapsulants, wherein their hydrophobic core could be used to hold a cargo of significant amounts (up to 20–30 wt.%) of water-insoluble encapsulates. At the same time, their hydrophilic corona

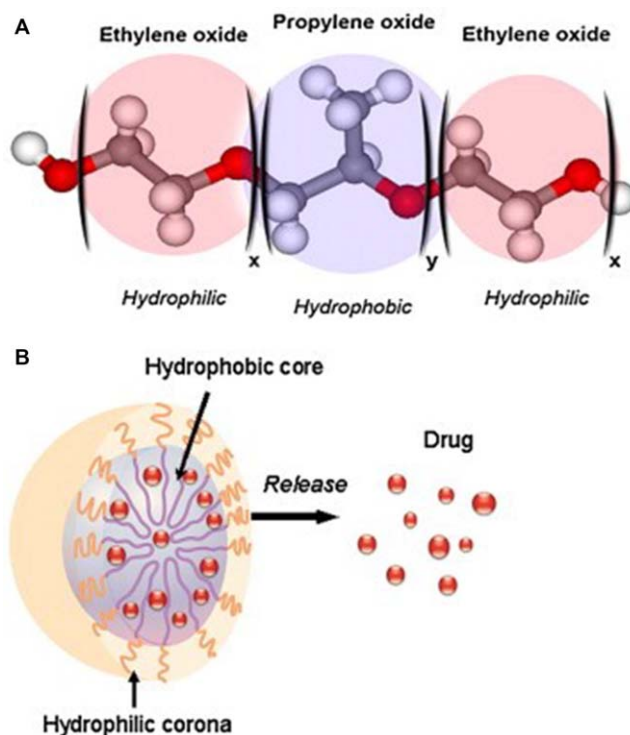


Figure 1.7 (A) A Pluronic block copolymer molecule. (B) A micelle with a solubilized drug in its core.

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allows for the micelles to exist in a dispersion, and thus reduces undesirable drug interactions with the biological system. It was thus thought that the incorporation of drugs into Pluronic systems could aid in increasing drug solubility and stability, and as a result bring about better drug pharmacokinetics and biodistribution. Furthermore, these systems offered advantages in specific applications such as anticancer chemotherapy, as the micelles enabled a passive drug-targeting route to tumors. This is due to the enhanced permeability and retention effect, which occurs due to the unusually high blood vessel permeability in tumors, as well as the extended circulation periods of the micelles within the body.²⁹ There was hence increasing amounts of focus being placed on Pluronics in the role of delivery vessels to meet a variety of medical needs.

The fabrication of an effective delivery system requires the understanding of the encapsulant-encapsulate behaviour to tune their properties as desired. Studies thus began to look into the effects solutes had on the Pluronic systems to better understand their behaviour in these interactions. Gilbert *et al.*, for example, inversely correlated solute diffusion coefficient with the concentration of Pluronic F-127 that could be described by an exponential equation: $y = 7213 \exp(-0.39 x) + 1.84$ ($r = 0.984$). They proposed that this result could be explained by an increased micelle size and number with increasing F-127 concentration, constricting the water channel sizes and increasing the path of diffusion.³⁰

Further understanding of the drug delivery thermogel systems was achieved by a study highlighting the effect solutes and polymers had on F-127's gelation properties. This investigation determined that the addition of solutes resulted in a concentration-dependent decrease in gelation temperature. Moreover, the inclusion of esters to the polymeric solution also decreased the gelation temperature possibly by binding to the polymeric chains, resulting in increased dehydration and hence increased micelle entanglement.³¹

The effect of salts and electrolytes on the micellization behaviour of the systems was also investigated. Urea addition increased the CMC and decreased enthalpy of micellization, with increasing urea concentration.²⁰ Studies such as these formed the basis for understanding these gels as drug delivery depots for other potential applications. Shortly after Schmolka's publication, a patent detailing the use of Pluronics thermogels as pharmaceutical vehicles for delivering drugs to mucous membranes was filed. This patent was concerned with a persistent problem in the treatment of ocular diseases, whereby aqueous solutions made poor contact when applied to mucous membranes, and this contact was not therefore sustained.^{32,33} Sustained release in ocular applications was difficult to achieve because solution-based dosage delivery could easily be diluted in the ocular tear film and spill over the lid margin.³⁴

Until a certain point in time, the polymeric carrier systems were used only as an inert tool that passively facilitated the delivery of actives, such as by offering protection, or sustained release. However, this notion changed upon

the discovery that certain synthetic polymers could induce a change in specific cellular response by acting as biological response modifiers. The Pluronic systems were found to be capable of this, acting to provide other benefits, such as sensitizing multidrug-resistant cancer cells, or promoting the transport of drugs across cellular barriers. The research, however, concluded that these functions were attributed to the polymeric unimers that are able to integrate into and translocate across the cellular membranes rather than the micelles associated with the thermogelling systems.²⁹ A thermogelling drug delivery system that can make use of this to its advantage might thus have to establish a finely-tuned balance between micellar aggregations used for drug transport and the unimers to elicit an appropriate response.

Although there has been much research directed into Pluronic drug delivery systems starting from early in its introduction, many of the studies can still be considered topical, and while injectable drug delivery depots involving the Pluronic systems have been known since the 1970s, subcutaneous use of Pluronic thermogelling systems was only reported much later.

1.5 Disadvantages of Pluronic Systems

The reason behind the lack of subcutaneous applications for Pluronic systems despite their reported biocompatibility can be attributed to several of their properties. Firstly, the Pluronic systems were not biodegradable³⁵ and were thus unable to be eliminated from the body after their temporary use. This resulted in side effects from accumulation, such as the toxic enhancement of triglycerol and plasma cholesterol.³⁶ Secondly, Pluronic gel systems demonstrated poor mechanical properties, and were easily eroded after administration.³⁵ It was found that the gel formed could not be stably maintained in a subcutaneous environment for more than a day, and hence limited its applications to short-term systems.²⁰ Later research efforts that were directed to designing biodegradable thermogelling systems with more suitable mechanical properties then paved the way for the Pluronic systems to be used in implant systems, which offered the benefits of minimally invasive entry *via* injection.

1.6 Modifications of Pluronic Copolymers

The disadvantages of Pluronic thermogels have necessitated their modification to fully exploit their potential in therapeutic devices. Research has seen the emergence of many Pluronic based systems that offer biodegradability and/or improved mechanical properties compared to the original gel systems.

1.6.1 Modified Pluronic Copolymers for Improved Mechanical Properties

With regards to achieving more desirable mechanical properties, many studies were aimed at allowing for the prevention of the rapid dissolution of

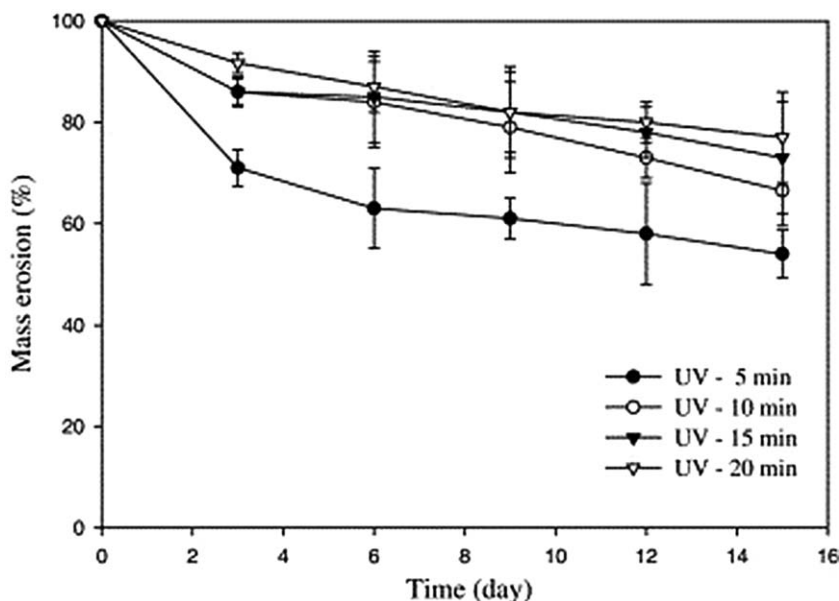


Figure 1.8 Degradation profiles of Pluronic hydrogels showing a reduction in mass loss with increased exposure to UV.

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Pluronic gels upon implantation. This instability of the hydrogels was thought to be attributed to the exposure of the gels to large solution volumes, which resulted in the immediate dilution of the polymer concentration and the consequent deterioration of gel structure and integrity (Figure 1.8).³⁷

The use of chemical crosslinks was thus one of the methods employed in combating this phenomenon. In a study by Chun *et al.*, hydrogels were formed through the photo-crosslinking of di-acrylated Pluronic macromers through UV irradiation. Prolonging the length of UV irradiation resulted in higher amounts of crosslinks within the gel, and allowed for a lower rate of degradation, which occurred through the cleavage of an ester linkage in the polymerized site. The effects of the increased crosslinking could also be observed in the decreased swelling of the gels. Furthermore, the improvement in mechanical properties of the gels could be observed through the increase in dynamic moduli values, which also increased with increased UV irradiation, compared to those of physical Pluronic hydrogels (Figure 1.9).³⁷

In another study making use of photo-crosslinking, supramolecular hydrogels that possessed highly elastic as well as thermoresponsive properties were developed through the use of a synthesized Pluronic F-68/poly(ϵ -caprolactone) block copolymer end-capped with acryloyl groups as a macromer with aqueous α -cyclodextrin (α -CD) for inclusion complexation.

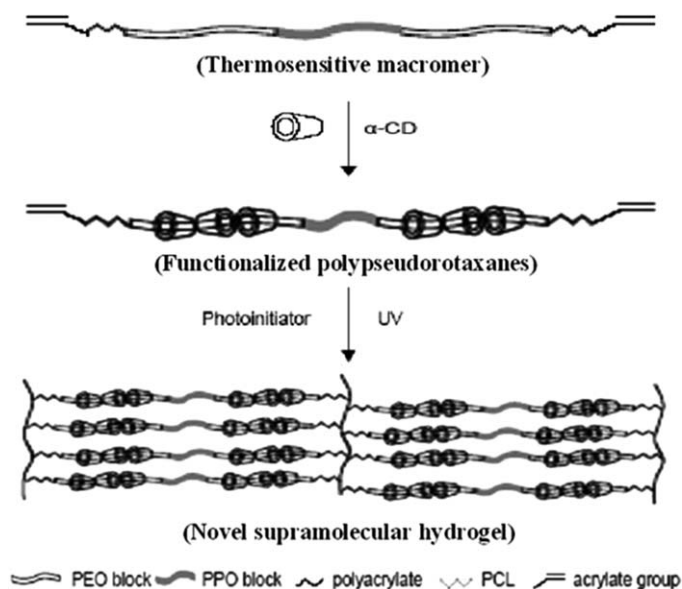


Figure 1.9 Illustration showing the formation of the novel supramolecular hydrogel with superior mechanical strength. Reprinted with permission from ref. 38. Copyright 2006 American Chemical Society.

This produced a physical hydrogel precursor with thermosensitive properties that could be tuned to the varying molar feed ratio of the two components. Upon *in situ* UV photo-crosslinking, the hydrogels displayed markedly improved viscoelastic properties with high elastic moduli.³⁸

Apart from photo-crosslinking, studies have also delved into the introduction of stereocomplexed crystalline domains within hydrogel structures to render them more physically stable. This was achieved through the inclusion of monomers such as poly(lactic acid) which possesses a chiral carbon atom and thus exhibits stereocomplexed crystalline behaviour. By linking multi-block Pluronic copolymers with D-lactide and L-lactide oligomers, stereocomplexed hydrogels capable of *in situ* formation could be formed through the mixing of the two enantiomeric copolymers. These hydrogels retained the temperature-responsiveness of Pluronic gel systems, and were shown to demonstrate increased mechanical strength through rheological assessments.³⁹

Improvement of Pluronic gel mechanical properties have also been reported by simply using it in blends with other polymers such as Carbopol or alginate. Studies observing a marked increase in gel strength when Pluronic was mixed with alginate attributed this to the formation of crosslinks between the polymers by virtue of water molecules acting as crosslinking agents and allowing for the formation of hydrogen bonds between the alginate carboxyl groups and the Pluronic ether groups.⁴⁰

The improvement of Pluronic gel stability and mechanical properties is crucial in anchoring its role in the biomedical industry. Without the ability to offer extended usage, the applicability of Pluronic systems in many biomedical issues that require long-term effects, such as in drug and gene delivery and tissue engineering, will be severely restricted.

1.6.2 Modified Pluronic Copolymers for Improved Biodegradability

Another property naturally lacking in Pluronic systems that is of utmost importance in the biomedical scene is biodegradability. Despite the rapid dissolution of the gel systems due to their instability, Pluronic systems cannot be processed, degraded and eliminated by the body. There have thus been numerous efforts to combat this problem in a variety of ways (Figure 1.10).

Early in 2002, it was shown that Pluronics could be employed as the hydrophilic segments in polyurethanes, with poly(ϵ -caprolactone) diol as the hydrophobic segment, to achieve polyurethanes with different hydrophobic-to-hydrophilic segment ratios that could undergo *in vitro* degradation with tunable rates. This resulted in mass loss, a lowering in the molecular weight and a decrease in polydispersity, as well as a loss of mechanical strength

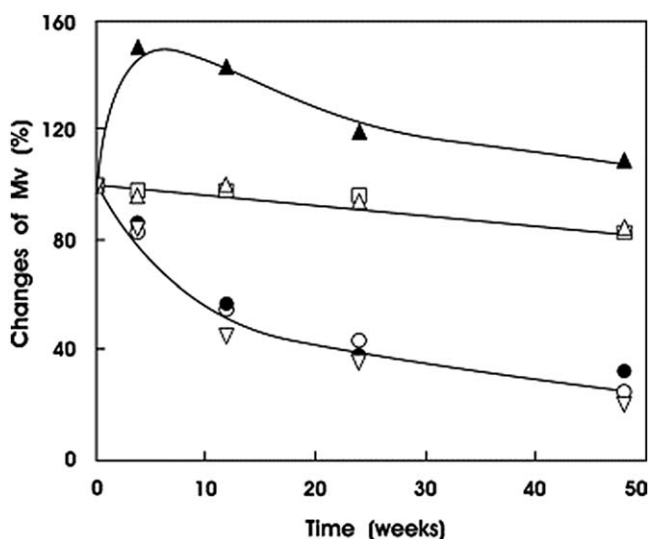


Figure 1.10 Plot of viscosity-average molecular weight (M_v) of the polyurethanes and controls against time of degradation. The polymers fabricated from mixtures Pluronic[®] and polycaprolactone (∇ , $\bullet$, $\circ$) saw a reduction of about 70–80% in M_v .

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with degradation. However, this work focused on the polyurethanes themselves, instead of a hydrogel system.⁴¹

A later study also performed in the early 2000s then saw the production of novel amphiphilic PLA-F127-PLA block copolymers through the grafting of poly(lactic acid) (PLA) onto both ends of Pluronic F-127. This resulted in the conferring of biodegradability to the hydrogel through hydrolytic degradation at two possible sites: in the middle of the PLA block and at the interface between the PLA and F-127 blocks. The copolymer described in this study, however, formed nanoparticles in PBS solutions, and did not maintain the stimuli-responsiveness of Pluronic gelling systems.⁴²

Further studies such as the one conducted by Liu *et al.*, which involved the development of amphiphilic PCL-Pluronic-PCL block copolymers through ring opening polymerization, saw greater success in retaining the thermosensitivity of the resulting gel systems while achieving biodegradability. Investigations made into the effects of varying the total molecular weight of the PCL block further revealed that the thermoresponsiveness and gelation requirements of the system could be tuned in such a way. By increasing that value, the team observed the lowering of the critical gel temperature, as well as the critical gel concentration, which effectively meant a wider temperature and concentration range for phase transition could be achieved to suit various purposes.⁴³

The stereocomplexed hydrogels described in the previous section, which made use of the linking of Pluronic copolymers by D-lactide and L-lactide oligomers to allow for improved mechanical properties through additional crosslinking brought about by their stereocomplex crystalline domains, were also proven to be biodegradable. This was made possible through hydrolytically cleaving the oligo(lactic acid) spacers that linked the copolymers. This system was proposed not only to achieve controlled protein delivery, but also for the possibility of subcutaneous and percutaneous delivery.³⁶

The constructs containing more F-127 displayed a considerably reduced size. As the amount of X-HA increased, the constructs carried a swollen morphology, seemingly due to the uptake of surrounding fluids. Only if an appropriate ratio was met could the composite hydrogel maintain its original size without a significant volume change.

The advent of tissue engineering, along with increased development of biodegradability and improved mechanical properties for Pluronic systems, brought about an increased interest in utilizing such systems as cell carriers and biocompatible scaffolds. Notably, Jung *et al.* demonstrated in 2010 the achievement of a composite hydrogel that made use of Pluronic F-127 derivatives and crosslinked hyaluronic acid with thermo-reversible gelling properties and biodegradability. Prior to this, studies investigating F-127 had shown it to have potential for providing an optimal 3D environment for cell growth and differentiation.⁴⁴ The Jung study, however, was novel in physically conjugating bioactive growth factors on to F-127 itself, instead of simply adding them in their free forms.⁴⁵

Although the use of Pluronics mainly as topical aids in the past has meant that biodegradability was a lesser concern compared with other properties, such as biocompatibility, growing demands to cope with today's needs are causing a rising importance in this property. The increasing research directed to achieving biodegradability has thus allowed Pluronic systems to be applicable in new areas.

1.7 Modern Applications of Pluronics

The applications we have looked at so far involve the modification of Pluronics to counter general problems that are largely faced in their biomedical usage. However, there has also been a large body of research directed at the using Pluronic systems to cope with specific and targeted needs (Figure 1.11).

For one, 3D printing has been a rapidly developing field in recent years, finding its footing in various fields. Notably, it has been applied in tissue engineering, and Pluronic copolymers have been one of the materials involved in serving as a bioink due to their thermo-responsive and rheological properties. Furthermore, although it was found that the high gelation concentration required for Pluronics caused reduced long-term cell viability, it was possible to make use of a high concentration only during printing and subsequently removing and reducing the concentration to allow for high cell viability in the printed prototypes (Figure 1.12).⁴⁶

Pluronics have also been additionally studied for use in cell printing applications due to their bio-inertness towards many cell types, their range of viscosities that induce lower cell stress, and the ability for them to be easily rinsed and removed post-printing through exposure to a temperature below their critical gelation temperature. They have thus been utilized in the bio-fabrication of cellularized hydrogel scaffolds, in which cells are first dispersed in the solution, and an additive-manufacturing printer can induce sol-to-gel phase transition and subsequently perform a layer-by-layer extrusion of the scaffold based on a prior computer design. This will thus form a cellularized construct for tissue engineering purposes.⁴⁷

With the advancement in modern technology, the smart properties of Pluronic thermogels may find a new range of applications, as there could be an increasing demand on selective material properties for emerging specific purposes.

1.8 Future Perspectives

Pluronic thermogelling systems have experienced increased and sustained attention over recent years because of their tunable thermogelling properties and biocompatibility. As efforts are made to overcome their disadvantages, there will be an expansion of their range of applications. They will then play an increasingly significant role in drug delivery. Originally thought to be rather inert vessels for delivery, Pluronic systems seem to have the potential

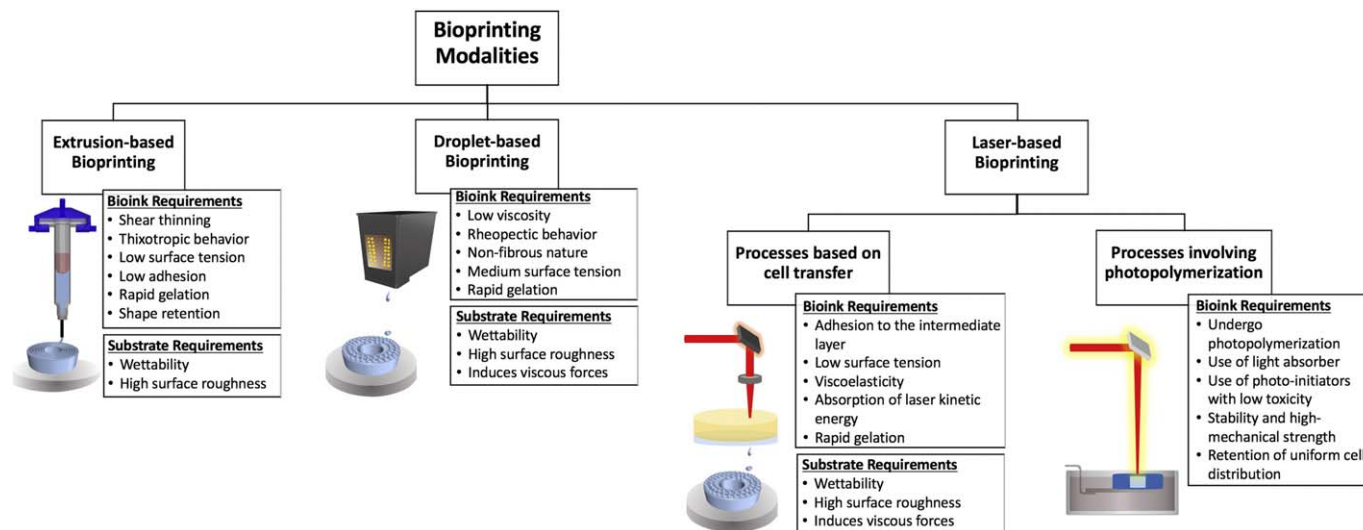


Figure 1.11 3D printed text a using nanostructured Pluronic approach.
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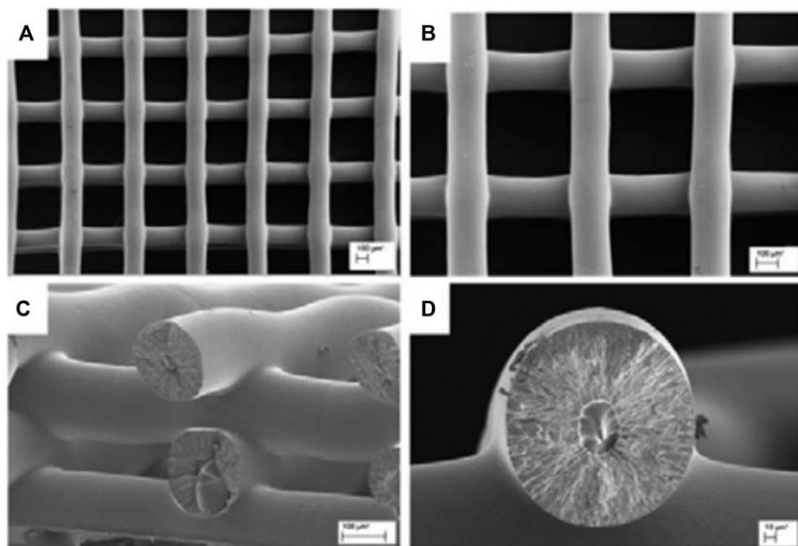


Figure 1.12 Micrographs of a hydrogel scaffold comprising of four layers captured through FEG-SEM.
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to play a nuanced role in controlled release.²⁹ In addition, recent studies have also sparked interest in their abilities to act as biological response modifiers, and in particular, aid in combating multidrug resistance in cancer therapy. This has placed Pluronic copolymers under the scrutiny of the nanomedicine lens, which is starting to play a more significant role in drug delivery. With this, their potential in areas such as tissue engineering and wound healing applications will be affected. However, a large portion of research seem to be looking into these properties independently of Pluronic systems' other benefits. Given that these hydrogels also offer stimuli-responsiveness, it will be interesting to observe if all of these advantages will in future be combined synergistically to solve medical problems.²⁹

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CHAPTER 2

Thermogelling PLGA-based Copolymers

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2.1 History and Structures

The initial incentive for developing thermogelling polymers was for drug-delivery. In the 1990s, polymers that responded to stimuli such as chemicals, temperature, pH and electrical field were actively investigated for controlled release of biological molecules such as insulin. Non-biodegradable thermosensitive polymers such as N-isopropylacrylamide and poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (PEG-PPG-PEG) inspired the search for biodegradable alternatives. Poly(ethylene glycol)-*b*-[DL-lactic acid-*co*-glycolic acid]-*b*-ethylene glycol (PEG-PLGA-PEG) was the first reported biodegradable thermosensitive block copolymer. There are various block structures for PLGA-PEG copolymers, including diblock, triblock, multiblock, and star-shaped block.¹ The chemical structure of a PEG-PLGA diblock copolymer is shown in Figure 2.1.

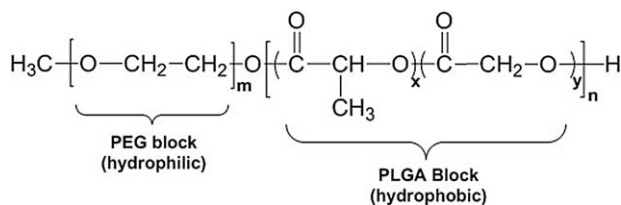


Figure 2.1 PEG-PLGA diblock copolymer.

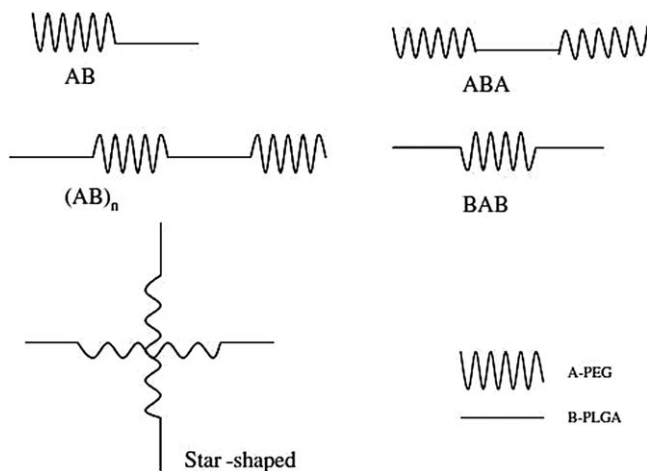


Figure 2.2 Diagrams of AB, $(\text{AB})_n$, ABA and BAB type PEG-PLGA copolymers. Reproduced from ref. 18 with permission from Elsevier, Copyright 2014.

PEG-PLGA thermogelling copolymers have four major configurations, namely AB type, $(\text{AB})_n$ type, ABA type and BAB type. “A” stands for the hydrophilic block PEG, while “B” is the hydrophobic block PLGA. AB type copolymers have two distinct sections of PEG and PLGA. $(\text{AB})_n$ type polymers have a number of repeating units of AB type. ABA type thermogel polymers have two hydrophilic A blocks at the two ends of the polymer chain and one hydrophobic B block in the middle section. Schematic representations of these block configurations are shown in Figure 2.2.

2.2 Synthesis

The copolymer can be synthesized by ring-opening polymerization of lactide and glycolide monomers with monomethoxypoly(ethylene glycol). PEG is used as an initiator of the reaction and stannous octoate as a catalyst.² Stannous octoate is the most popular catalyst,^{3–5} although a wide variety of other main group and transition metal catalysts have been investigated. The triblock copolymers (PEG-PLGA-PEG) are synthesized by coupling another PEG block onto the diblock copolymers using hexamethylene diisocyanate⁶ (Figure 2.3).

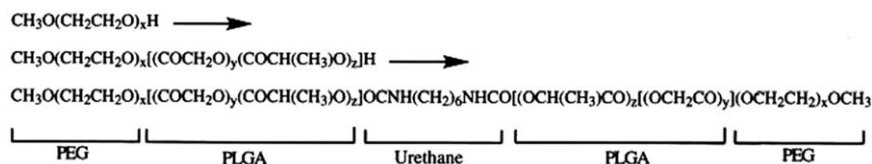


Figure 2.3 Synthesis of PLGA-PEG block copolymers.

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2.3 Properties

2.3.1 Reversible Sol-to-gel Transition

The gel-to-sol transition for PEG-PLGA copolymers was first discovered by Jeong *et al.* in 1997.⁶ They reported that the diblock and triblock copolymers form gels at 45 °C but go through a gel-to-sol transition when the temperature decreases to body temperature. The sol-to-gel transition at temperatures lower than body temperature was discussed in their work in 1999. PEG-PLGA-PEG (550-2810-550) transits from sol to gel in the temperature range 30–35 °C and gel to sol in the range 40–70 °C.⁷ The complete phase diagram for the reversible sol-to-gel transition is presented in Figure 2.4. The transition temperature is concentration dependent and the critical gel concentration is 16 wt% (Figure 2.4).⁷ Concentrations of the block copolymers in water also influence the transition temperature. An increase in the concentration of the polymer solution increases the temperature gap between the sol-to-gel and gel-to-sol transitions, making the gel phase more stable.

The mechanism of the reversible sol-to-gel transition behavior is due to the amphiphilic nature of PLGA-PEG block copolymers (Figure 2.5). The polymers can form micelles in water, and during micelle formation hydrophobic B blocks collapse into the core while hydrophilic A blocks are still solvated by water and swell and extend at the periphery of the micelle.

This micelle formation has been confirmed by ¹³C-nuclear magnetic resonance (NMR) spectroscopy (Figure 2.6). The PLGA signal collapses and broadens in D₂O compared to the sharp peak in CDCl₃, which is a good solvent for both PEG and PLGA. This contrast demonstrates the poor solvation of PLGA by D₂O. As a result, PLGA collapses and the ¹³C nuclei relax more quickly, resulting in a signal that reduces in intensity and broadens in width.

Micelle formation depends on the concentration of PLGA-PEG polymer. The critical micelle concentration (CMC) has been determined by UV-vis absorption spectroscopy in the presence of hydrophobic 1,6-Diphenyl-1,3,5-hexatriene dye (DPH) (Figure 2.7). DPH absorbs light at 365 nm. The solubility of DPH in water is rather low. However, when the concentration of PLGA-PEG polymer increases, DPH has higher chance of being captured inside the hydrophobic core of the micelles. When stronger absorption at 365 nm starts to occur the CMC has been reached.⁷

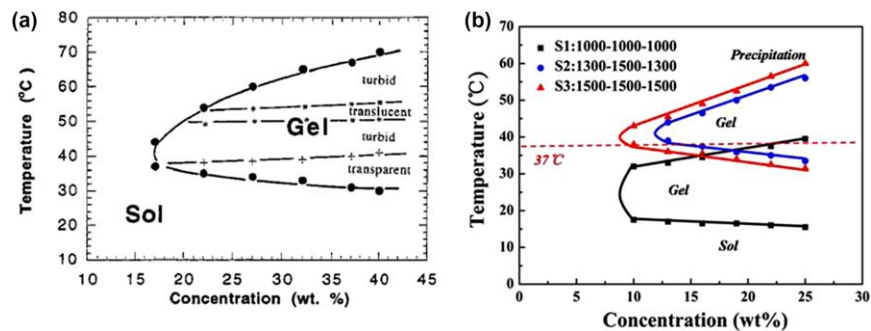


Figure 2.4 Reversible sol-to-gel transition phase diagrams for (a) PEG-PLGA-PEG (550-2810-550);⁷ (b) PDLLA-PEG-PDLLA copolymers. Reproduced from ref. 2 under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>, Copyright © 2016 Macmillan Publishers Limited.

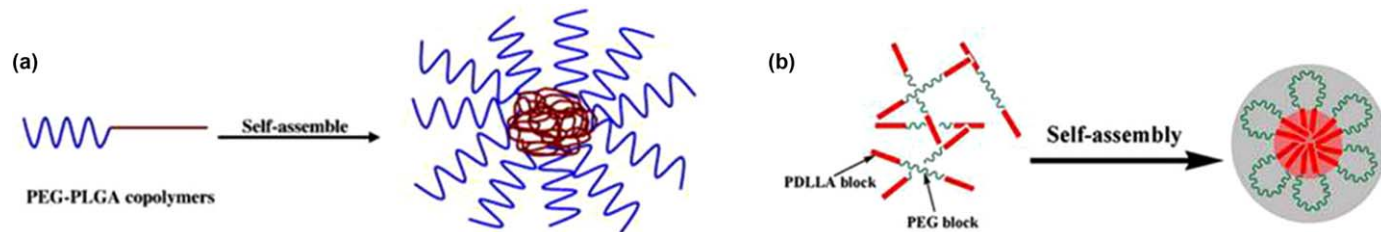


Figure 2.5 (a) AB type micelle; (b) BAB type micelle. Part a reproduced from ref. 18 with permission from Elsevier, Copyright 2014. Part b reproduced from ref. 2 under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>, Copyright © 2016 Macmillan Publishers Limited.

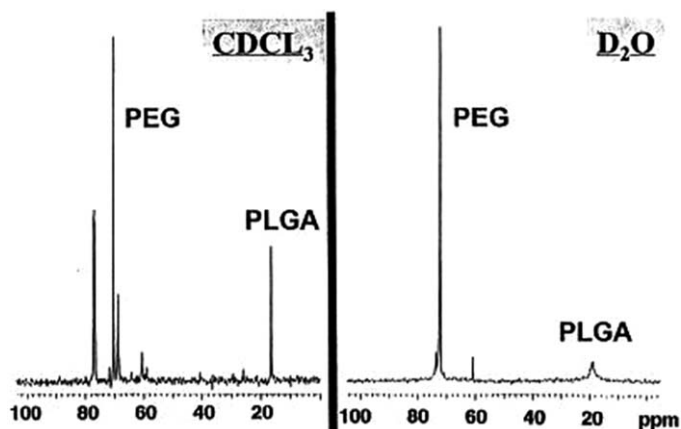


Figure 2.6 ^{13}C -NMR spectrum of PLGA-PEG Block copolymer in CHCl_3 and D_2O . Reproduced from ref. 7 with permission from Elsevier, Copyright 1999.

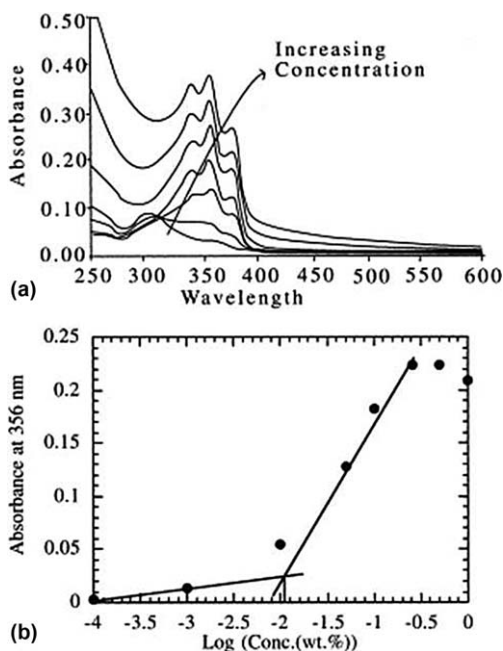


Figure 2.7 UV-vis absorption spectra of hydrophobic DPH dye. (a) Increasing concentration of PLGA-PEG polymer. (b) Determining CMC *via* extrapolation of absorbance *versus* polymer concentration. Reproduced from ref. 7 with permission from Elsevier, Copyright 1999.

Micelle formation strongly depends on the segment lengths and the length ratio of PEG and PLGA. Longer PEG chain segments result in higher solubility of the triblock copolymers and higher CMCs.⁷ As shown in

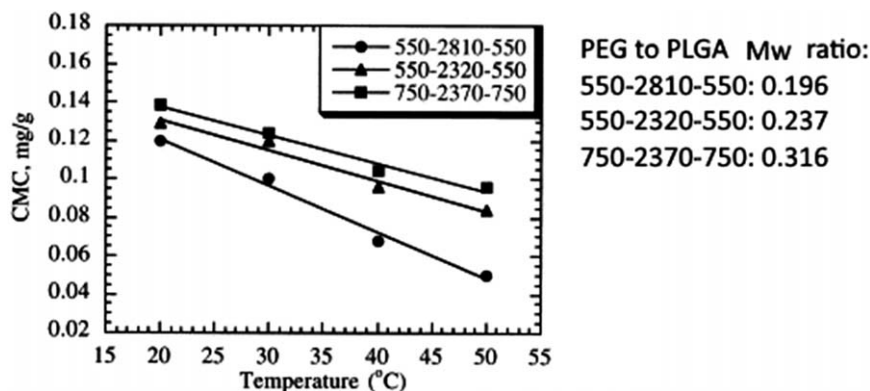


Figure 2.8 CMC dependency on temperature and PEG to PLGA molecular weight ratio.

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Figure 2.8, the larger the PEG to PLGA molecular weight ratio, the higher the CMC at the same temperature.

Solubility of PLLDA-PEG-PLLDAs copolymers is poor when the M_n ratio of PLLDA and PEG is above 1. The best ratio to generate a sol-to-gel transition near to body temperature (*i.e.* between 10 and 60 °C) appears to be 0.5. These copolymers are of research interest since their transition behavior is applicable to biological systems. When the M_n for PEG is above 1500, the triblock copolymers show no sol-to-gel transition in the temperature range of interest.²

2.3.2 Degradation

PLGA-based copolymers undergo hydrolysis *in vivo*. It is important to understand the degradation behavior of PEG-PLGA hydrogels for applications such as drug delivery and postoperative adhesion barriers.

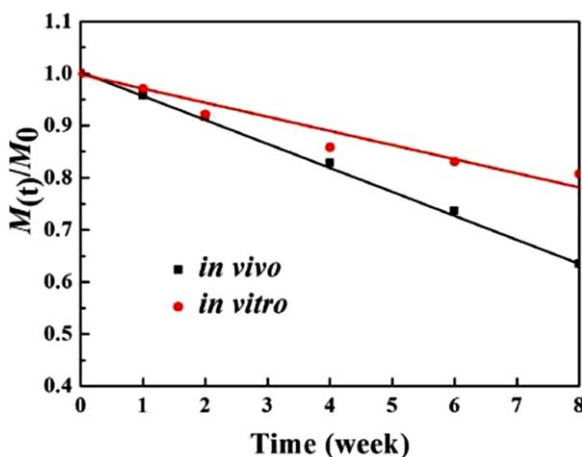
2.3.2.1 *In Vitro*

Significant swelling (100%) of PDLLA-PEG-PDLLA hydrogels occurs in the first 2 weeks after immersion in PHB (pH = 7.4) buffer solution. The pH drops to about 6.4 and by week 8, the hydrogel completely loses its shape and dissolves into the buffer solution.² Gel permeation chromatography (GPC) results show that the molecular weight decreases to 85% of the original after 8 weeks.

PLA-PEG hydrogel shows significant *in vitro* degradation within 4 weeks according to the GPC results.⁸ The molecular weight reduces by more than 50% (Table 2.1). A higher PEG to PLA ratio corresponds to faster degradation rate because hydrophilic PEG facilitates water penetration and erosion.⁸

Table 2.1 PLA/PEG ratio and molecular weight. Reproduced with permission from ref. 8.

Multiblock copolymer	Feed ratio (unit ratio) (PLA/PEG)	Composition (unit ratio) ^a (PLA/PEG)	Molecular weight ^b		
			Mw	Mn	Mw Mn ⁻¹
LE(m)-16	85/15	84/16	85 300	45 900	1.86
LE(m)-32	70/30	68/32	83 200	46 300	1.80
LE(m)-53	50/50	47/53	73 400	40 400	1.82
LE(m)-88	14/86	12/88	42 400	26 500	1.60

^aMeasured by NMR.^bMeasured by GPC.**Figure 2.9** *In vivo* degradation of PDLA-PEG-PDLA.

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Degradation slows down from week 4 to 12. After 12 weeks, the molecular weight of the samples reduces to less than 20% of the original.

2.3.2.2 *In Vivo*

In vivo degradation is faster than that *in vitro*. Molecular weight drops to about 60% of the original in 8 weeks and the hydrogel is completely absorbed by week 10 (Figure 2.9).²

2.3.3 Biocompatibility

PEG-PLGA block copolymers demonstrate viable biocompatibility. In the case of PDLA-PEG-PDLA, the viability test for fibroblasts and vascular endothelial cells (L929 and human umbilical vein endothelial cells (HUVEC)) gave above 85% cell survival even at high concentration (2.5 mg ml⁻¹).²

The PLGA-PEG-PLGA polymers synthesized *via* ring-opening with a molecular weight of 1500 Da gave rise to a cell viability of over 80% when tested against MC3T3 cells in the culture medium with polymer concentrations as high as 10 mg ml⁻¹.⁹ Hemolysis percentage was below 5% for the concentration range from 0.1 to 10 mg ml⁻¹ for PDLLA-PEG-PDLLA.² Immunological response tests on PDLLA-PEG-PDLLA triblock copolymers on BALB/c mice have demonstrated no long-term damaging effect.² Acute inflammation occurs in the first week after implantation and slowly reduces into mild chronic inflammation in weeks 2 to 6. After 10 weeks of implantation, the thermogel is completely dissolved and the surrounding tissues rehabilitate with no chronic damage.

2.4 Applications

2.4.1 Drug Release

Thermogels are considered promising candidates for controlled, long-term drug release applications. The PEG-PLGA-PEG system was first developed primarily for delivery of high- M_r protein drugs and sustained release of low- M_r hydrophobic drugs. The elimination of organic solvents in drug encapsulation using thermogels (compared with previous drug-delivery systems) protects sensitive protein drugs from denaturation.⁶

2.4.1.1 Mechanism and Release Profiles

PEG-PLGA thermogels carry drugs within the micelles, either physically trapped in the hydrophobic core or covalently bonded to the polymer chain. The physical entrapment of the drug is generally easier and leads to higher loading rates, while the chemical coupling confers the polymer-drug system a higher stability upon injection.¹⁰ The release mechanism differs depending on the hydrophilicity and the molecular weight of the drug. In general, drug release involves either diffusion or a combination of diffusion and erosion.⁴ Small, hydrophilic drugs are released mainly by diffusion since they are located in the extended hydrophilic area of the micelles. Hydrophobic drugs encapsulated inside the core of the micelle can be released *via* erosion of the thermogel. Large protein-based drugs are released *via* erosion due to their low diffusivity.

The drug release profile is influenced by many factors, including concentration of the copolymers, unit ratio of PEG to PLGA blocks, pore size, degradability, drug concentration, and the chemical or physical interactions between the drug and the copolymers. Higher concentration of polymer in the solution gives rise to slower release (Figure 2.10a).⁴ However, too high a concentration of the polymer leads to lower transparency, higher osmolality, and faster gelling kinetics, which may not be desirable in certain applications.

Hydrophilic drug release is mostly diffusion controlled (*e.g.* Ketoprofen in PEG-PLGA-PEG (Figure 2.10a). Hydrophobic drugs, on the other hand,

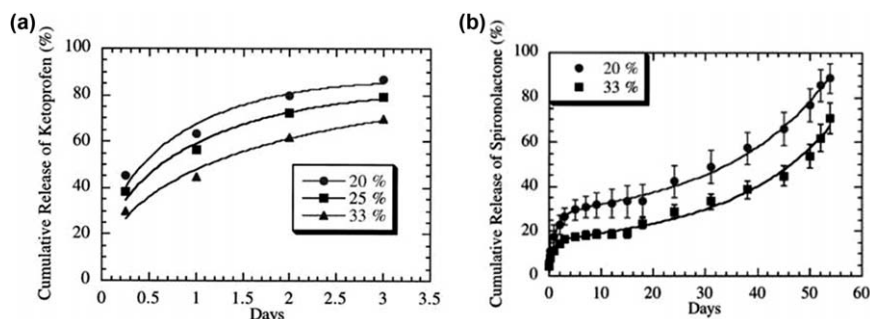


Figure 2.10 (a). Ketoprofen release profile (legends specify the concentration of the copolymers in solution). (b) Spironolactone release profile (legends specify the unit ratio of PEG-PLGA-PEG blocks).

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follow a different release mechanism. Thermogels with longer PLGA blocks can encapsulate more hydrophobic drugs, and the release is slower (Figure 2.10b).

Mathematical models for hydrophilic and hydrophobic drug release profiles have been proposed by Jeong *et al.*⁴ Diffusion-drug release can be modelled by eqn (2.1):

$$M_t/M_\infty = 1 - \sum \{8 \exp[-D(2n+1)^2 \pi^2 t/l^2] / (2n+1)^2 \pi^2\} \quad (2.1)$$

In the initial stage, eqn (2.1) can be approximated by eqn (2.2):

$$M_t/M_\infty = 4(Dt/\pi l^2)^{1/2}, \quad M_t/M_\infty \leq 0.6 \quad (2.2)$$

Noteworthy is that the cumulative release amount is proportional to $t^{1/2}$, as predicted by Fick's Law. In the later phase of the release profile, the approximation of eqn (2.1) yields eqn (2.3):

$$M_t/M_\infty = 1 - 8/\pi^2 \exp[-\pi^2 Dt/l^2], \quad M_t/M_\infty > 0.4 \quad (2.3)$$

Hydrophobic drugs show a more complicated release profile due to the two-step release mechanism. Spironolactone in PEG-PLGA-PEG, for example, exhibits an S-shaped release profile (Figure 2.10b),⁴ modelled by eqn (2.4):

$$dM_t/dt = B \exp(-kt) + C[\{\exp(k't)\}/t]^{\frac{1}{2}} \quad (2.4)$$

The modelled profile (Figure 2.10b, as solid lines) gives a reasonable fitting of the experimental results. The release profiles of PEG-PLGA-PEG hydrogels are in general non-linear. However, in clinical applications, a zero-order or linear release profile is more desirable. A study with calcitonin as a model protein delivered from a PLGA-PEG-PLGA hydrogel claims to have achieved zero-order release for up to 100 hours.⁹ However, the release profile reported appears non-linear (Figure 2.11).

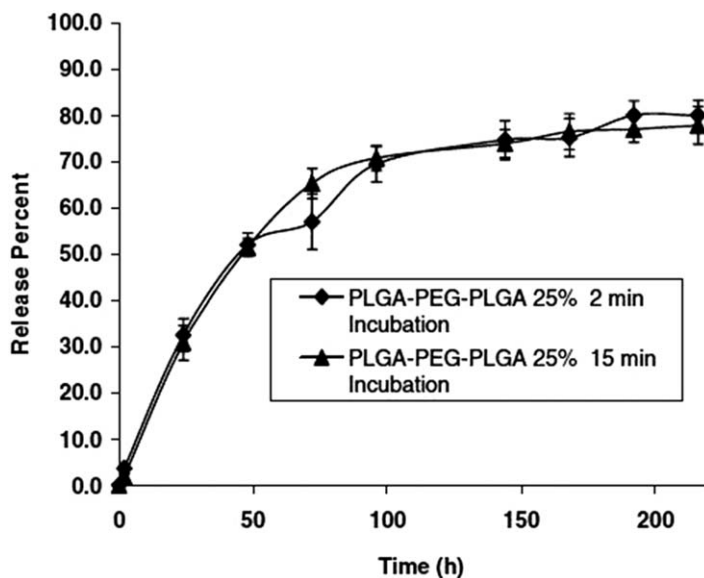


Figure 2.11 Calcitonin release profile from PLGA-PEG-PLGA thermogels. Reproduced from ref. 9 with permission from Taylor and Francis Ltd.

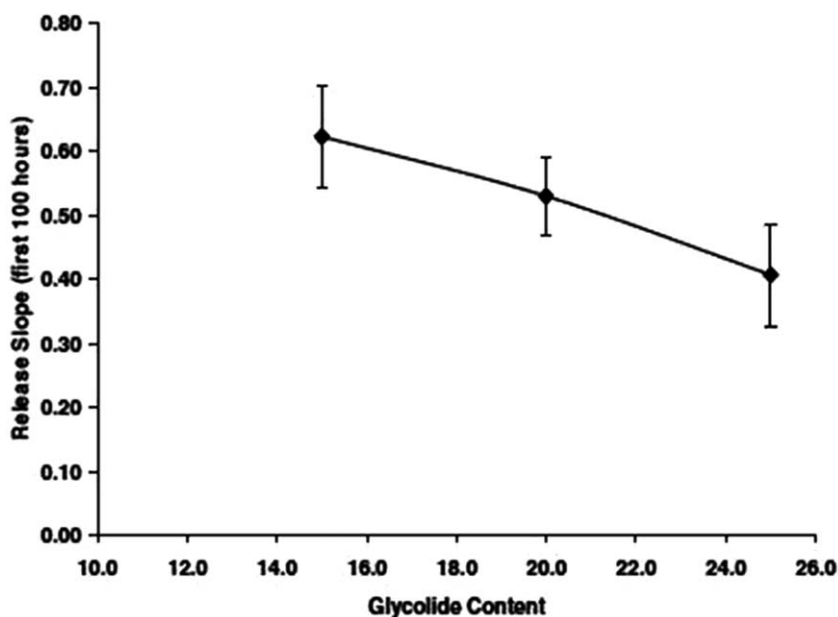


Figure 2.12 Calcitonin release rate and glycolide content. Reproduced from ref. 9 with permission from Taylor and Francis Ltd.

The initial rate of release corresponds to the glycolide percentage of the PLGA block. The higher the content of hydrophobic glycolide, the slower the release (Figure 2.12).

2.4.1.2 Anti-cancer Drugs

PEG-PLGA based hydrogels have been shown to be effective for delivering anti-cancer drugs such as irinotecan (IRN). IRN is an anti-tumor drug approved by the US Food and Drug Administration³ and can be loaded into PLGA-PEG-PLGA micelles *via* a two-step dissolving procedure. Firstly, the drug is dissolved into saline at 85 °C and then the polymer is dissolved into the solution at room temperature with magnetic stirring. The *in vitro* release profile can be fitted by eqn (2.5) (Figure 2.13):

$$\frac{M_t}{M_\infty} = kt^m \quad (2.5)$$

In vivo testing in a nude mouse model bearing human SW620 colon tumors has shown significant reduction in tumor weight with the tumor inhibition ratio about 20% higher than the IRN control group (delivered *via* injection of drug saline solution).³ The tumor volume also decreased after an initial increase. In comparison, the tumor volume for the IRN/saline group continued to increase slowly. The sustained release of IRN *via* PLGA-PEG-PLGA thermogel also resulted in milder side effects. Two possible side effects were investigated, namely weight loss and white blood cell loss. No significant weight loss was observed for the IRN/thermogel group. White blood cell loss was less significant than for the IRN/saline group and recovered to normal after 8 days of implantation.³

Another anti-cancer drug, doxorubicin (DOX), has been incorporated into PLGA-PEG-PLGA thermogels for prolonged release.¹¹ PLGA-PEG-PLGA (1675-1500-1675), which has a LA/GA molar ratio of 10/1, was used as the drug carrier. Drug loading was achieved *via* mixing at room or lower temperature. Drug concentrations up to 2 mg ml⁻¹ had no significant influence on the viscosity of the thermogel. However, when concentration increased to

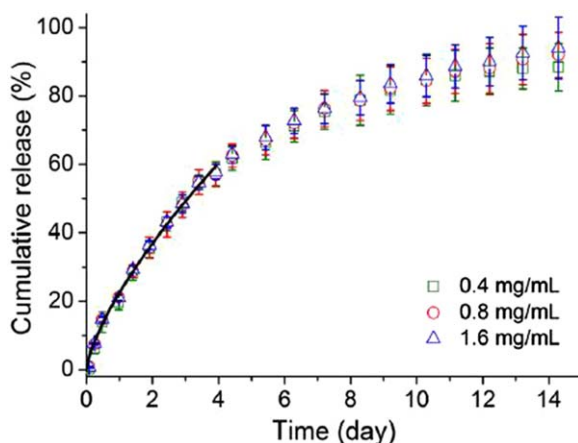


Figure 2.13 IRN *in vitro* release profile from a PLGA-PEG-PLGA hydrogel. Reproduced from ref. 3 with permission from Springer Nature.

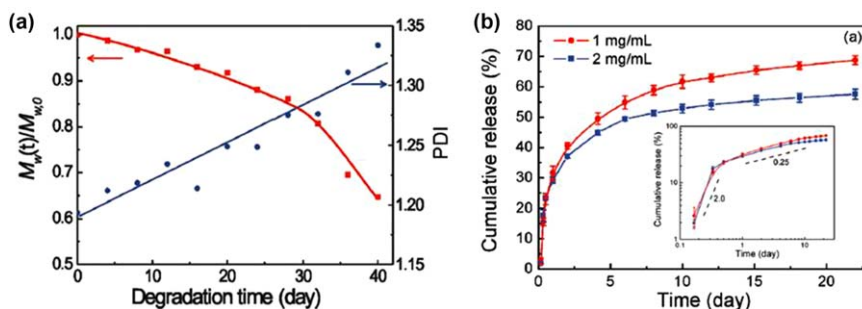


Figure 2.14 (a) PLGA-PEG-PLGA (1675-1500-1675) *in vitro* degradation. (b) Release of DOX in PBS buffer solution. Reproduced from ref. 11 with permission from The Royal Society of Chemistry.

4 mg ml^{-1} , injection of the thermogel was hindered by the increased viscosity. *In vitro* degradation and release profiles of PLGA-PEG-PLGA (1675-1500-1675) are shown in Figure 2.14. Burst release was observed over the first 3 days.

Anti-cancer efficiency was studied with S-180 bearing KM mice. The DOX/thermogel system gave rise to a 7% higher tumor inhibition ratio compared to the DOX/solution system with the same dose (10 mg ml^{-1}). Weight loss in the DOX/solution group was about 2 g more than for the DOX/thermogel group.¹¹ The overall efficiency of the DOX/thermogel system appears to be less attractive than the IRN/thermogel system. However, the DOX/thermogel system offers a more convenient solution for long-term drug release compared with the DOX/solution system, which requires multiple injections.

2.4.1.3 Diabetes

Researchers have investigated incorporating insulin and other drugs for diabetes treatment into thermogels since the 2000s. ReGel™ is a commercialized PLGA-PEG-PLGA thermogel shown to constantly release human insulin in male Zucker Diabetic Fatty (ZDF) rats for up to 14 days¹² (Figure 2.15a). Zinc carbonate (10 wt%) was added into the hydrogel solution to reduce the initial burst release. The zinc complex also helped stabilize the polypeptide. The blood plasma insulin level was monitored after one injection of 1 ml insulin formation (Figure 2.15b). The composition of the insulin formation was 23 wt% PLGA-PEG-PLGA, 6 mg ml^{-1} insulin and 10 wt% zinc carbonate. The blood glucose levels in ZDF rats (Figure 2.16) was significantly lower than the usual 80 to 120 mg dl^{-1} and was stable over the 4- to 8-day period after injection. Although the author claimed that there was no burst release and the injection could function for 1 week, we can see that the initial insulin level rises in the first 3 days and the blood glucose level drops to around 50 mg dl^{-1} on Day 3. This fluctuation may not be desirable for patients.

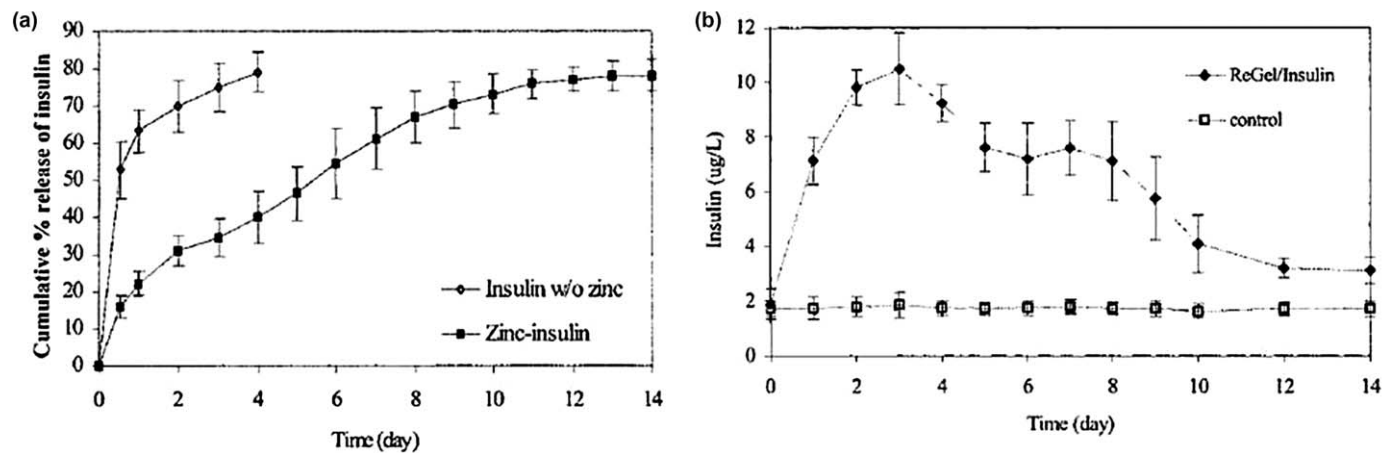


Figure 2.15 (a) Zinc-complexed insulin/ReGel™ *in vitro* release profile. (b) Plasma insulin level. (labeled a and b). Reproduced from ref. 12 with permission from Springer Nature, © Plenum Publishing Corporation 2003.

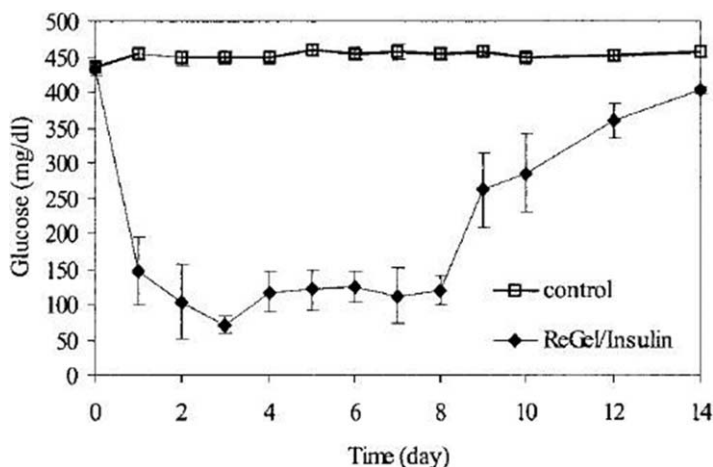


Figure 2.16 Blood glucose level in ZDF rats. Reproduced from ref. 12 with permission from Springer Nature, © Plenum Publishing Corporation 2003.

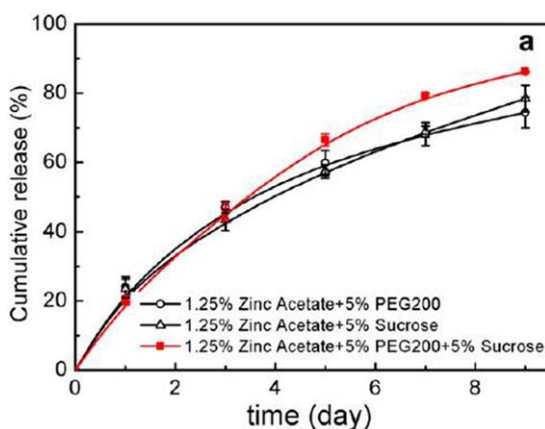


Figure 2.17 The effect of additives on the EXT release profile in 25 wt% PLGA-PEG-PLGA (1286-1500-1286 mixed with 1508-1000-1508, mix ratio 1 to 1). Reproduced from ref. 13 with permission from Elsevier, Copyright 2013.

To further control the initial burst release, researchers have examined various additives in the drug/thermogel system. The addition of 1.25% zinc acetate, 5% PEG200, and 5% sucrose stabilizes the polypeptide drug exenatide (EXT), release profile during the first week (Figure 2.17).¹³

A fatty acid-modified antidiabetic polypeptide, liraglutide, was later investigated as a model drug in PCGA-PEG-PCGA(poly (ϵ -caprolactone-co-glycolic acid)) & PLGA-PEG-PLGA.¹⁴

2.4.2 Gene Delivery

PLGA-PEG-PLGA copolymer can assist endocytosis of PEI-DNA polyplexes and thus enhance gene transfection efficiency. Five- to ten-fold increments of gene transfection were obtained in the presence of the PLGA-PEG-PLGA tri-block copolymer at a low concentration of 0.25% (w/v), compared with Pluronic F68 at the same concentration range.⁵ Hepatoma cell line (HepG2), fibroblast (NIH3T3), and smooth muscle (A7R5) cell lines were investigated. Although different cell lines demonstrated various level of transfection, all PLGA-PEG-PLGA copolymer-coupled polyplexes displayed higher transfection levels than the control groups. The polymer-PLL-DNA polyplex system has also been investigated as a delivery vehicle for the miL-18 gene.¹⁵ Polycationic peptide polylysine (PPL) was used to increase the gene concentration before PEG-PLGA-PEG was added to form the polyplex. The degradation rate of the polyplexes varied with different PLA to PGA ratio: the larger the lactide to glycolide ratio, the slower the degradation. Slower degradation was desirable for this application because high concentrations of lactide and glycolide acids was toxic to the surrounding cells. PEG-PLGA-PEG with a PLA to PGA ratio no smaller than 75 : 25 is consistent with the US Pharmacopoeia XXIII requirements for cytotoxicity. These polyplexes with cisplatin were also found to be useful in the treatment of lung cancer in mice. The tumor inhibition rate for the PPDs + Cisplatin group was about 20% higher than the group given purely cisplatin treatment.

2.4.3 Postoperative Adhesion Prevention

PEG-PLGA block polymers and their derivatives can be used as postoperative peritoneal adhesion barriers.^{2,8,16,17} PDLLA-PEG-PDLLA hydrogel was shown to be a better choice for adhesion prevention than the commercial hyaluronic acid (HA)-based adhesion barrier.² Two weeks after the sidewall defect and bowel abrasion operation, rat models without adhesion barriers had developed score 3 adhesions.[†] Most of the HA-treated rats developed score 1 to 3 adhesions. However, in the PDLLA-PEG-PDLLA-treated group, only one out of eight models suffered from adhesion. All the others had no adhesion and the wounds healed within 2 weeks.²

In a comparative study of various adhesion barrier materials, PLGA-PEG-PLGA triblock polymer gave the best adhesion prevention after 1 month.¹⁷ PLGA-PEG-PLGA (1675-1500-1675), PCGA (poly(caprolactone-co-glycolide)) – PEG-PCGA and PCL (polycaprolactone) – PEG-PCL, and chitosan were applied onto the wound of rabbits after sidewall defect-bowel abrasion. After 1 month, half of the PLGA-PEG-PLGA group showed a score of 0 adhesions, the other half scored 1. The groups for the other treatment methods had at least

[†]The scores for adhesions are defined as: score 0, no adhesion; score 1, mild intestinal adhesion and separation was facile; score 2, moderate intestinal adhesion and blunt dissection was needed; score 3, severe intestinal adhesion and sharp dissection was needed.¹⁷

one score 3 adhesion sample and a few others with score 2 adhesions. These results indicate that PLGA-PEG-PLGA hydrogel is a more effective choice for postoperative adhesion barriers.

PLA-PEG hydrogel as anti-adhesion barrier has also been investigated in rat models prepared by scrubbing the cecum with abrasive paper and removing the pericardium of the heart.⁸ The researchers developed a double-layered film made of LE(m)-32 (PLA to PEG unit ratio is 70/30) and LE(m)-88 (PLA to PEG unit ratio is 14/86) (Table 1, Section 3.2). LE(m)-88 with higher PEG had higher hydrophilicity and thus was used as the inhibitor for protein adsorption and cell adhesion. LE(m)-32 with lower hydrophilicity prevented LE(m)-88 from being washed away by blood and fixed the film in place. This double-layered film demonstrated promising results. All of the seven heart models scored 0 adhesion after 1 week.

2.5 Areas for Future Research

Burst release appears to be the most common issue that inhibits the use of PEG-PLGA thermogels as drug release carriers. As demonstrated in Figures 2.14 and 2.15, the initial release rate (within first 4 days) is much larger than during the following period. Sustained slow release is obviously more desirable for clinical applications such as insulin administration. Understanding the mechanism of burst release, therefore, and developing methods for prevention are areas for future investigation. The mechanism of PEG-PLGA-thermogel-mediated gene delivery is still somewhat ambiguous. The two examples described in Section 4.2 both indicated that polyplexes improved gene transfection efficiency, but exactly how is unclear. Likewise, the observation that tumor inhibition is higher with PPD-mediated cisplatin chemotherapy is also worthy of further study. The mechanism of postoperative adhesion prevention by PLGA-PEG-PLGA hydrogels will guide the future search for adhesion barrier materials. Comparison of PLGA-PEG-PLGA, PCGA-PEG-PCGA, and PCL-PEG-PCL hydrogels as postoperative adhesion barriers indicates that PLGA-PEG-PLGA is most effective material. However, the reasons why remain unclear. Although PCL-PEG-PCL hydrogel degrades more slowly and retains inside rabbit models for longer, this hydrogel shows the poorest adhesion prevention.¹⁷

2.6 Conclusions

PEG-PLGA-PEG is one of the most promising thermogelling polymers for biomedical applications. It undergoes sol-to-gel transition at 30–35 °C and has good biocompatibility. Degradation takes 8 to 10 weeks and generates mild degradation products. The major application for PEG-PLGA-PEG thermogels is in drug delivery. The two most clinically relevant applications investigated so far are cancer and diabetes treatments. Additionally, thermogels can be used to facilitate gene delivery and for postoperative adhesion

prevention. The mechanism and prevention of burst release during thermogel-mediated drug delivery is arguably the most pressing question to be addressed in this rapidly growing field.

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CHAPTER 3

Polyester-based Biodegradable Thermogelling Systems as Emerging Materials for Therapeutic Applications

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3.1 Introduction

Hydrogels are soft materials made from hydrophilic homo-polymers or co-polymers, and have three dimensional (3D) cross-linked network matrices with pore sizes of several micrometers.^{1–5} They can absorb water up to many thousands of times their weight, and this property results in good biocompatibility.^{3,6–9} Similarly, they can also behave like reservoirs to store drugs or macromolecules for targeted and timed drug delivery.² The chemical and physical properties of hydrogels can be tuned easily by changing the

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synthetic methods and components. They can be chemically stable or designed to dissolve depending on the type of cross-linking.¹⁰ Hydrogels are, therefore, excellent candidates for various biomedical applications, such as tissue engineering, controlled protein and/or drug delivery, and as adhesives.^{11–13}

Hydrogels can be formed *in situ* in response to environmental stimuli, such as change of temperature, pH, light and ionic strength.^{14,15} They can be categorized depending on the nature of the cross-linking in the gel. Chemical *in situ* gels, also called “permanent” gels, are hydrogels containing covalently cross-linked networks. They are irreversible polymeric networks formed with cross-linking reagents or enzymes.¹⁶ Physical *in situ* gels, or “reversible” gels, are networks held together by molecular entanglements and secondary forces such as hydrogen bonding, and ionic and hydrophobic forces.^{17,18} These weak, reversible bonds depend on external stimuli to form.^{19–22} An example is a thermogel. Temperature-responsive hydrogels form reversible, physical cross-links upon heating to the critical temperature. They have a number of therapeutic applications.^{23–25} An injectable thermogel system can be used for pharmaceutical formulations by mixing in the drug below the sol-to-gel transition temperature. This can avoid the need for surgical implantation. After injection, body temperature will stimulate sol-to-gel transition and the viscous solution will solidify into a drug delivery depot. The high percentage water content makes the thermogel highly compatible with the injection site. Peptides are kept at low temperature before application to reduce denaturation. There are currently only two commercial thermo-responsive hydrogel products on the market. One of these is Amphipathic Poloxamer, a US Food and Drug Administration (FDA)-approved triblock polymer composed of hydrophobic poly(ethylene oxide) (PEO) and hydrophilic poly(propylene oxide) (PPO) components.²⁶ This hydrogel has been widely used in both therapeutic and consumer applications. The other is Pluronic F127, a type of Poloxamer, with a critical micelle temperature (CMT) at body temperature (37 °C). Although Poloxamer based hydrogels are biocompatible and used for drug delivery,²⁷ the degradation rate of 4 hours has limited their use for long-term drug release applications.¹² Poly(*N*-isopropyl acrylamide) (PNIPAAm) is another typical amphipathic thermo-responsive polymer, first synthesised in the 1950s.^{28–30} This hydrogel can undergo a reversible phase change on altering the temperature of the aqueous environment. However, poor mechanical and biodegradable properties have limited its applications.³¹ PNIPAAm-based hydrogels need to be removed by surgery after their drug delivery role is complete.

Polyesters are one of the earliest and most studied class of biomaterials.^{32–38} Polyesters have three advantages over comparable biomaterials: biodegradation, biocompatibility and synthetic versatility.^{39–45} The application of polyester-based hydrogels as a scaffold for cell transplantation benefits thousands of patients every year.⁴⁶ This biodegradable polymer can be made with a high molecular weight, and is able to undergo

hydrolytic and enzymatic degradation. This is due to its highly hydrolysable ester linkages that fit the appropriate esterase active sites.^{24,38,47–49} Controllable degradation gives polyester-based hydrogels an advantage for periodic and targeted drug release.⁵⁰ The end products of degradation are carbon dioxide and water. Moreover, polyester-based hydrogels can trap a large amount of water, including body fluids. Another unique property of polyester-based hydrogels is their structural diversity. They are made by polycondensation of diols and dicarboxylic acids, self-polycondensation of hydroxy acids, and by ring-opening polymerization of lactones and lactides.^{51,52} An ideal polymeric thermogelling system for biomedical application should have a responsive range near physiological temperature, a reasonable biodegradation rate, enough mechanical strength for its intended application, biocompatibility, good targeting specificity and synthetic versatility for property tuning. Polyester-based thermogelling systems are one of the best candidates to satisfy these requirements.

3.2 Polyester-based Thermogelling Systems

3.2.1 The Poly(lactic acid)-based Thermogelling Systems

Poly(lactic acid) (PLA)-based materials are popular choices among the new biomedical materials.^{32,40,44,45} They are biocompatible, biodegradable and made from renewable resources.⁴⁴ The constitutional unit of PLA is lactic acid (2-hydroxypropanoic acid, $\text{HOCH}_2\text{CHCOOH}$), which is a chiral molecule existing as L- and D-enantiomers. There are two stereocenters of lactic acid and so numerous isomeric polymers and crystal structures have been reported.⁵³ This polymorphism gives scientists freedom to blend different PLA isomers and adjust their composition ratios to get modulation of the crystallinity for any specific application. Stereo-complexes of poly(L-lactic acid) (PLLA) and poly(D-lactic acid) (PDLA) are highly crystalline isotactic polymers. They have better physical properties than their diastereomeric counterparts for many applications, Poly(D,L-lactic acid) (PDLLA) is an amorphous, atactic polymer with a higher melting point and stronger mechanical properties.^{44,53} Block copolymers of PLA and poly(ethylene glycol) (PEG) can undergo a thermo-reversible sol-to-gel transition.⁴⁴

The common L-isomer of PLA is a brittle material, unable to resist high-level stress. It also has poor thermal stability.⁴⁴ However, many strategies have been developed to improve these deficiencies, such as plasticization, copolymerisation and melt blending with flexible polymers.^{13,53–57}

An alternating PLLA/PEG block copolymer can be made by condensation of corresponding polymers (Figure 3.1a) and undergoes thermogelling in aqueous solution above ≥ 15 wt%.⁵⁸ This sol-gel-sol transition is reversible and occurs in the temperature range 20 to 60 °C. Nuclear magnetic resonance (NMR) spectroscopy, dynamic light scattering (DLS) and dye solubilisation results indicate that gelation is due to micelle aggregation. These micelles grow larger in size by trapping water molecules and promote the

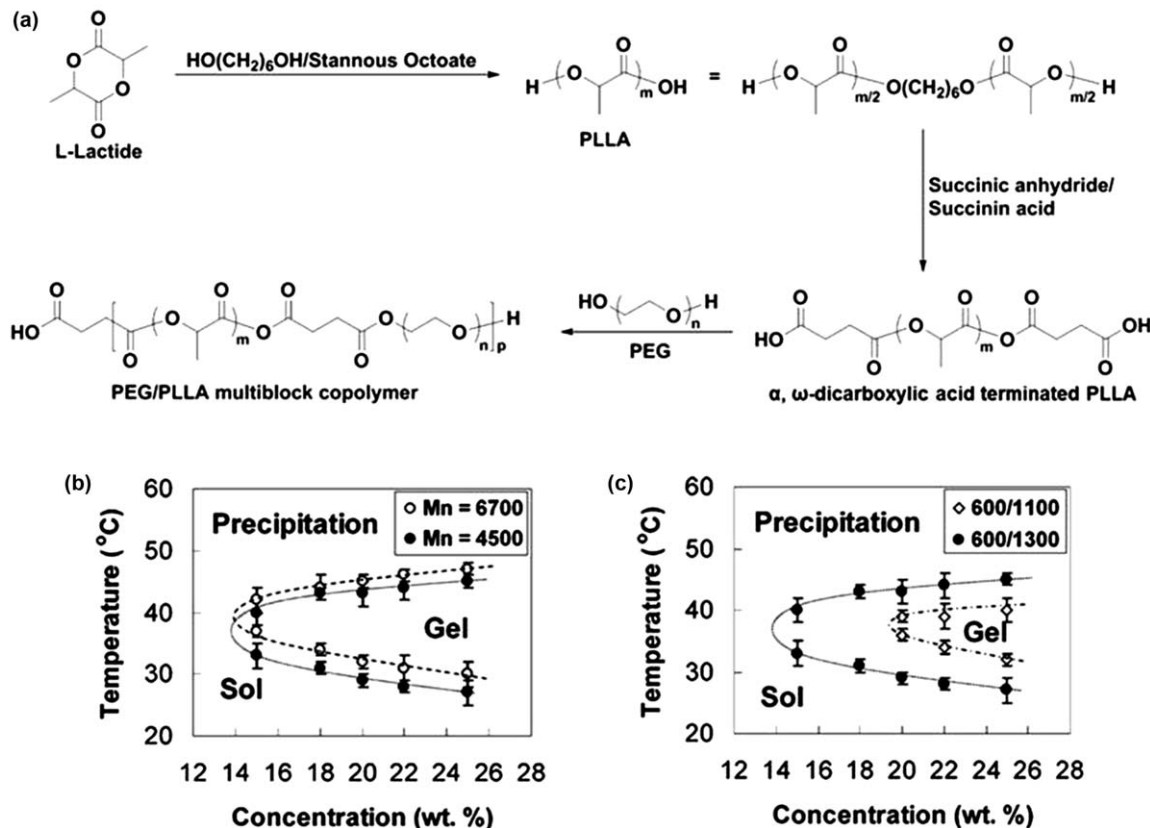
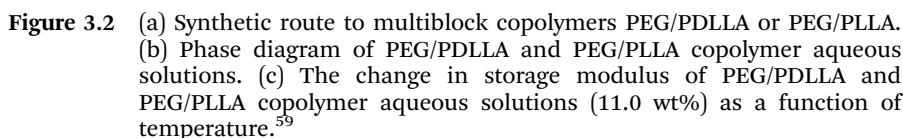


Figure 3.1 (a) Synthetic route to PEG/PLLA multiblock copolymers. (b) Effect of total molecular weight of PEG/PLLA (600/1300) copolymers ($M_n \approx 4500$ and 6700) on the sol-to-gel transition of their aqueous solutions. (c) Effect of PLLA length of PEG/PLLA copolymers (600/1100 and 600/1300) on the sol-to-gel transition of their aqueous solutions.⁵⁸ Reprinted with permission from ref. 58. Copyright 2006 American Chemical Society.

The influence of the stereochemistry of PLA multiblock copolymers on reversible thermal gelation has been studied by Jeong and co-workers.⁵⁹ Aqueous solutions of PEG/PDLLA and PEG/PLLA multiblock copolymers with similar block lengths and molecular weight were prepared, and their sol-to-gel transition performance observed. PEG/PLLA had the lower critical gel concentration and sol-to-gel transition temperature. Additionally, the maximum gel modulus of PEG/PLLA was three times that of PEG/PDLLA (Figure 3.2). The further investigation by NMR, critical micelle concentration (CMC), hydrophobic partition and circular dichroism indicated that this stereo-isomeric variation of the thermogel is due to the lower dynamic molecular motion and intrinsically higher aggregation tendency of isotactic PLLA.



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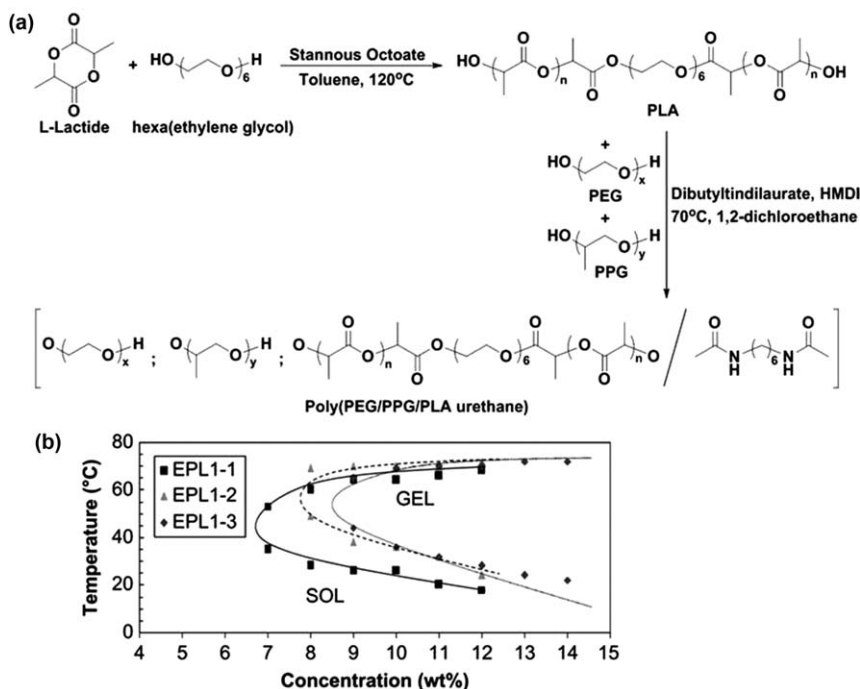


Figure 3.3 (a) Synthetic route to PLA-diol and poly(PEG/PPG/PLA urethane)s. (b) Sol-to-gel phase diagrams of Poly(PEG/PPG/PLA urethane)s (EPL1) thermogels in aqueous solutions.⁶⁰ Reproduced from ref. 60 with permission from Elsevier, Copyright 2008.

In 2008, Loh and co-workers synthesized hydrolytically degradable and thermo-responsive copolymers containing PEG, poly(propylene glycol) (PPG) and PLLA blocks, using hexamethylene diisocyanate (HMDI) as a coupling agent (Figure 3.3a). The critical gelation concentration (CGC) of aqueous solutions of prepared copolymers was relatively low, ranging from 7 to 9 wt% (Figure 3.3b). The diluted aqueous copolymer solutions displayed a lower critical solution temperature (LCST).⁶⁰

3.2.2 Polycaprolactone-based Thermogelling Systems

Polycaprolactone (PCL) is an extensively studied biodegradable crystalline polymer, which can be easily synthesized at low cost. It stands out from many counterparts for biomedical applications because of its good rheological and viscoelastic properties, good biocompatibility and low immunogenicity.^{8,25,37,61–63} The degradation products of PCL can be metabolized by the human body, and PCL has been approved by the FDA for use in surgery. PCL performs better in drug release applications than PLA or polyglycolic acid (PGA) because of high permeability to drugs and less acidic degradation products.⁶⁴ However, PCL properties, such as high crystallinity,

inherent hydrophobicity and lack of reactive groups along the PCL backbone, limit its other biomedical applications. High crystallinity results in PCL undergoing a plastic deformation when stretched by external forces. Its application in active soft tissues is therefore restricted. PCL's inherent hydrophobicity results in poor cell-scaffold interaction and prevents homogeneous distribution of therapeutic molecules into the scaffolds.⁶⁵ Slow degradation is also a resultant effect of this high crystallinity and hydrophobicity.⁶⁶ Hence, researchers have investigated various methods to functionalize PCL to overcome these limitations, such as blending with other functional materials,^{65,67,68} polymerization of caprolactone with other monomers containing reactive moieties, termination of living PCL chains, and manipulating chemical cross-links and physical associations in and between crystalline domains.⁶⁹ These modifications allow tuning of PCL's mechanical properties.

In 2001, Lee and co-workers firstly synthesized a series of thermo-responsive and biodegradable PEG/PCL copolymers by one-step random condensation copolymerisation.⁷⁰ The CGC and an upper phase-transition temperature of their aqueous solutions could be altered by adjusting the PEG/PCL block ratio, PEG or PCL block length and molecular weights. A phase separation-induced gelation mechanism was proposed to explain the reversible thermo-responsive transition. These PEG/PCL copolymers were fine-tuned so that at body temperature, they were able to undergo sol-to-gel transition and therefore could potentially be used in an injectable drug delivery system. A series of water-soluble multiblock copolymers PEG-PCL-PEG were synthesized by Jeong and coworkers in 2005 (Figure 3.4a).⁷¹ The crystalline nature of PCL gave the final triblock copolymer brittle

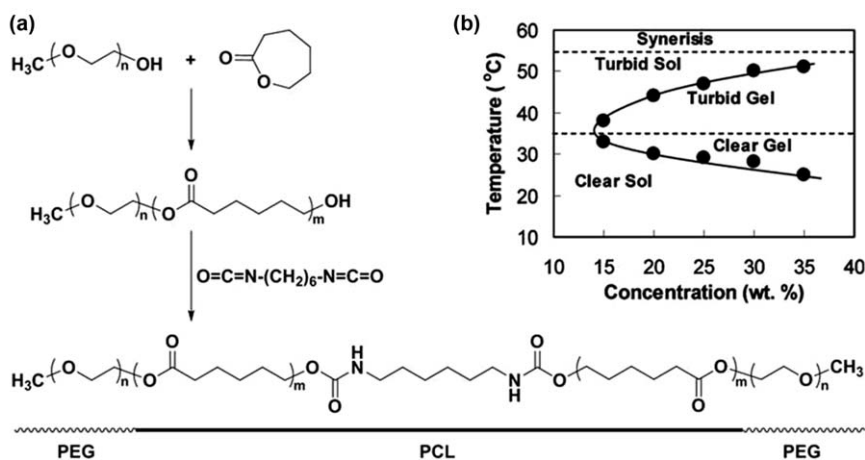


Figure 3.4 (a) Synthetic route to the PEG-PCL-PEG triblock copolymers. (b) Phase diagram of PEG-PCL-PEG triblock copolymer in deionized water.⁷¹ Reprinted with permission from ref. 71. Copyright 2005 American Chemical Society.

powder morphology in addition to its thermogelling property, which made the copolymers easier to handle and faster to dissolve in water. Aqueous solutions of these copolymers (>15 wt%) underwent a closed loop “clear sol-gel-turbid sol” transition over the temperature range 20 to 60 °C (Figure 3.4b). Investigation by DLS and NMR spectroscopy indicated micellar aggregation as the sol-to-gel transition mechanism, while increased molecular motion of PCL was responsible for the gel-to-turbid sol transition. PCL is also approved by the FDA as a contraceptive implant. Hence, PEG-PCL-PEG copolymers are very promising materials for various applications, such as drug delivery, cell therapy and tissue engineering.

In the same year, this group also reported PCL-PEG-PCL triblock copolymers.⁷² This material was easier to make compared with PEG-PCL-PEG copolymers, because it avoided the HMDI coupling step and, in addition, the gel weight percentage for the aqueous solutions enjoyed a larger range of 15–32 wt% (Figure 3.5c), and a larger gel modulus. An aqueous solution of PCL-PEG-PCL was temperature responsive and able to undergo sol-to-gel transition over the range 10 to 60 °C. The effect of PCL and PEG length on the phase diagram has been investigated (Figure 3.5a and b) and elucidates the mechanism of the sol-to-gel transition. PCL-PEG-PCL has a lower

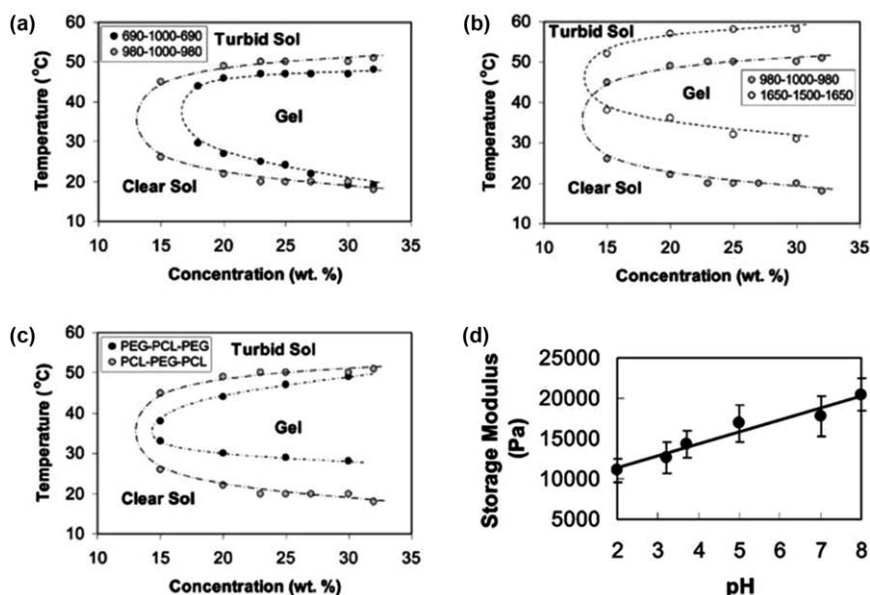


Figure 3.5 (a) Effect of PCL length and (b) effect of PEG length on the phase diagram of PCL-PEG-PCL triblock copolymer aqueous solutions. (c) Effect of topology on the phase diagram of triblock copolymer aqueous solutions. (d) Effect of pH on the gel modulus of PCL-PEG-PCL triblock copolymer (690-1000-690) at 37 °C. The gel shows maximal modulus in a pH range of 2–8.⁷²

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sol-to-gel transition temperature, wider gel window and higher modulus than PEG-PCL-PEG (Figure 3.5c). The influence of pH on the gel modulus of PCL-PEG-PCL triblock copolymer (690-1000-690) at 37 °C has also been investigated (Figure 3.5d). Both types of thermogel are water soluble and biodegradable. They show promising therapeutic possibilities, and both exist in a powder form instead of sticky semi-solids and so are easier to handle.

Later, Lee and co-workers synthesized a series of pH and temperature, dual responsive multiblock poly(ester amino urethane)s ((denoted as (PCL-PEG-PCL-PAU)_n) by condensation polymerisation (Figure 3.6a).⁷³ The tertiary amino groups of the poly(amino urethane) (PAU) blocks in (PCL-PEG-PCL-PAU)_n copolymers imparted pH-responsiveness, while the PCL-PEG-PCL blocks were temperature sensitive and biodegradable. Aqueous solutions of these copolymers maintained their sol state under acidic conditions, and displayed a sol-to-gel-to-aggregation transition with increasing temperature under neutral or basic conditions (Figure 3.6b). By varying the hydrophilic/hydrophobic balance and block length, the critical gelation pH value and sol-to-gel phase diagram could be adjusted to suit the requirement of the application.

In 2008, Loh and co-workers synthesized a series of novel temperature-sensitive triblock copolymers with two ended hydrophilic PNIPAAm blocks linked to a central hydrophobic PCL block, using atom transfer radical polymerization (ATRP) (Figure 3.7).⁷⁴ These copolymers were water soluble due to the hydrophilic PNIPAAm blocks of varying lengths, and the well-defined triblock polymeric structures made possible by ATRP. Core-shell micellization and the micellar characteristics of the copolymer aqueous solutions were investigated systematically, and their CMC fell in a very low range of 4–16 mg l⁻¹. The phase transition behaviour of these copolymers at LCST indicated temperature sensitivity. This behaviour was tunable by varying the composition of the copolymers.

In 2009, Lee and co-workers carefully designed and synthesized a series of pH and temperature dual sensitive PAE-PCL-PEG-PCL-PAE pentablock copolymers for a fundamental study of factors affecting sol-to-gel transition in aqueous solution (Figure 3.8).⁷⁵ Weight ratios of 20–30 wt% underwent sol-to-gel transition with changes in pH and temperature. The sol-to-gel transition window could be finely tuned by varying the PCL/PEG block ratios (hydrophobic/hydrophilic blocks), PEG molecular weight, Poly(β-amino ester) (PAE) molecular weight (pH sensitive block) and the copolymer concentrations. This material was used to develop a sustained, injectable delivery system for insulin.⁷⁶

Hyperbranched amphiphilic multiblock copolymers poly(PPG/PEG/PCL urethane)s (HBPECs) have been synthesized from PPG-diol, PEG-diol, and PCL-triol units using HMDI as a coupling agent (Figure 3.9).⁷⁷ This material displayed thermo-responsive micelle formation and aggregation behaviour. The hyperbranched structure and associated urethane linkages provided more hydrophobic and hydrogen bonding sites than its linear counterparts.

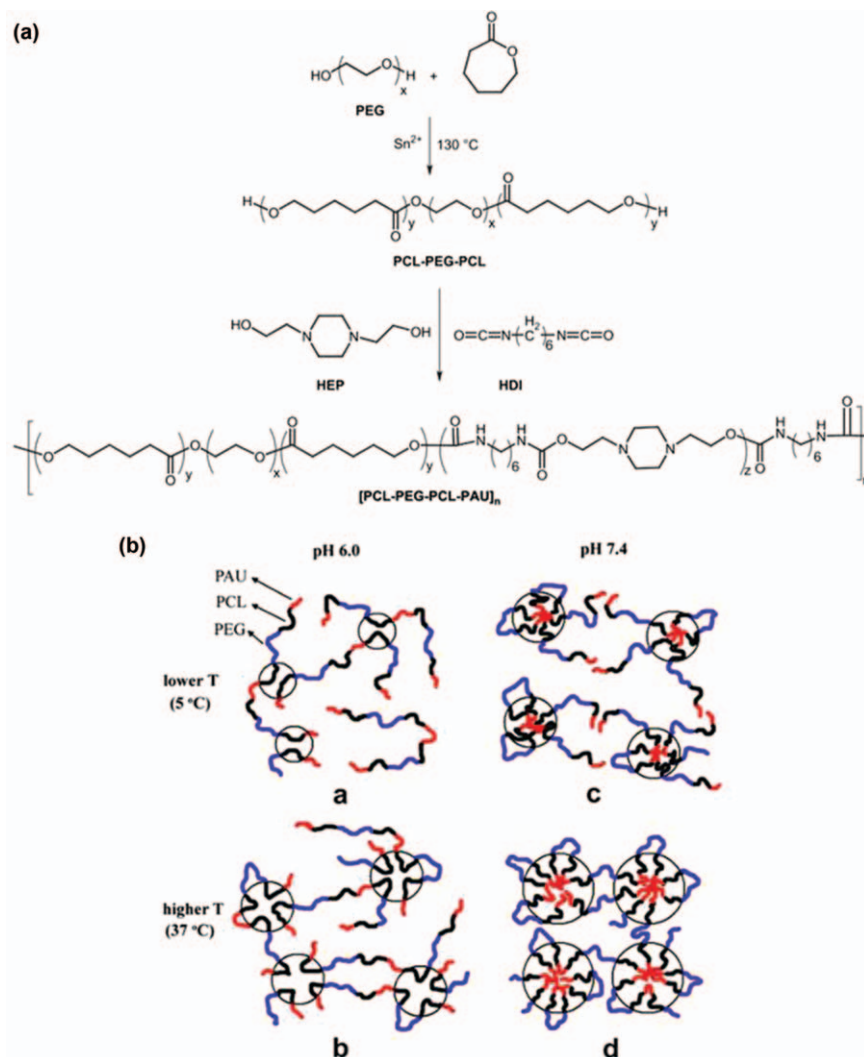


Figure 3.6 (a) Synthetic route to the copolymers $(\text{PCL-PEG-PCL-PAU})_n$. (b) Schematic illustration of the sol-to-gel phase diagrams of $(\text{PCL-PEG-PCL-PAU})_n$ copolymer aqueous solutions.⁷³ Reproduced from ref. 73 with permission from Elsevier, Copyright 2008.

Similarly, HBPEC had a much lower LCST value than its linear counterpart poly(PPG/PEG/PCL urethane) (LPEC) copolymer, and much lower CGCs, ranging from 4.3 to 7.4 wt%, than the value for LPEC or Pluronic F127 (PEG-PPG-PEG).⁷⁷ HBPEC formed a dehydrated gel instead of a turbid gel at high temperature (Figure 3.10). This behaviour is normally associated with linear thermogels. Biodegradability and good biocompatibility for HBPEC was demonstrated by hydrolytic degradation and cytotoxicity studies.

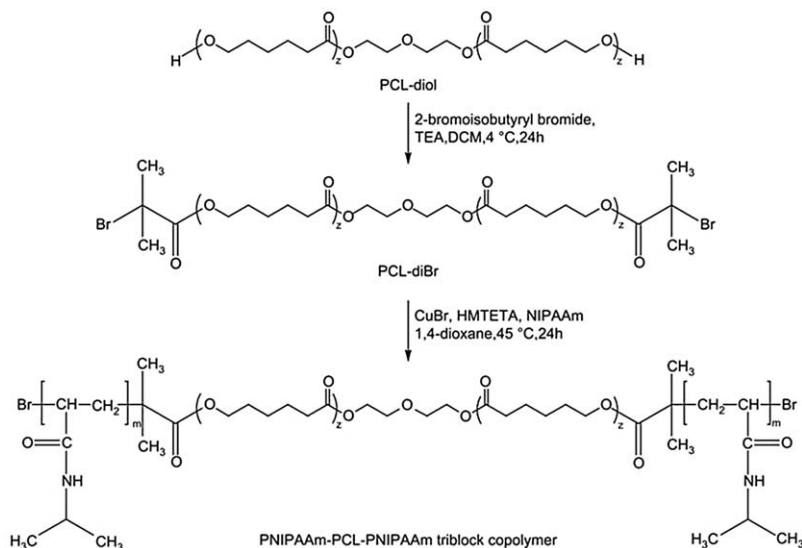


Figure 3.7 Synthetic route to the PNIPAAm-PCL-PNIPAAm triblock copolymers.⁷⁴ Reproduced from ref. 74 with permission from Elsevier, Copyright 2008.

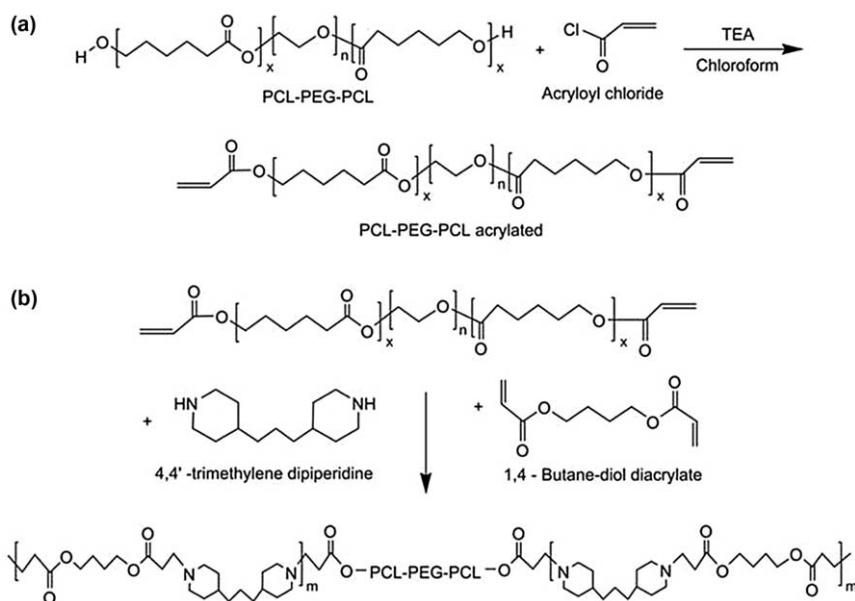


Figure 3.8 Synthetic routes to (a) acrylated PCL-PEG-PCL triblock copolymer and (b) PAE-PCL-PEG-PCL-PAE pentablock copolymer.⁷⁵ Reproduced from ref. 75 with permission from Elsevier, Copyright 2009.

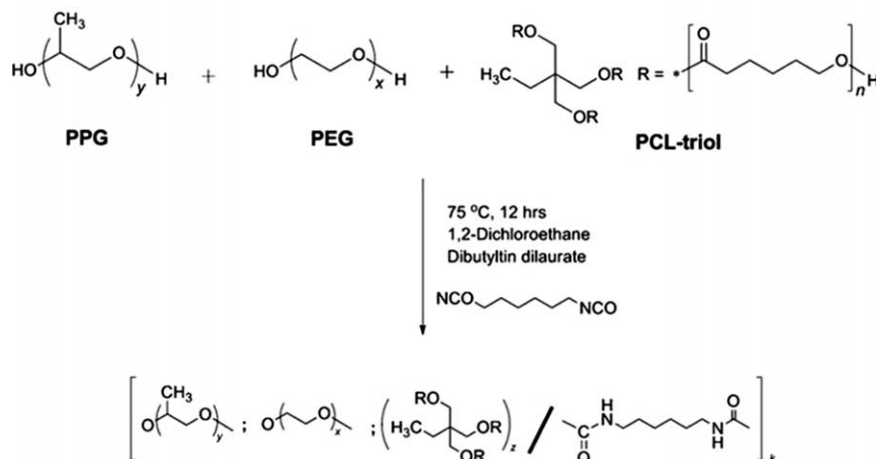


Figure 3.9 Synthetic route to HBPEC block copolymers.⁷⁷

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3.2.3 Poly([R]-3-hydroxybutyrate)-based Thermogelling System

Poly([R]-3-hydroxybutyrate) (P3HB), also called PHB, is one of the most common types of polyhydroxyalkanoate (PHA) with three carbon monomers in the backbone.^{9,43,78,79} PHAs are a family of biodegradable polyesters and make up the largest group of natural polymers.⁸⁰ More than 150 different types of PHA monomers have been identified, and are synthesized by microorganisms for structural and energy storage purposes.^{35,81} PHAs are made biochemically by unbalanced growth during fermentation and at the accumulation stage as part of a survival mechanism of the microbes.⁸² PHAs are generally either short-chain-length, 3–5 carbon monomers (e.g. 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), or medium-chain-length with 6–14 carbon monomers (e.g. 3-hydroxyhexanoate (3HHx), 3-hydroxy-octanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD)) (Figure 3.11).^{9,83 84}

Although PHAs are natural, abundant polymers, they have some disadvantages that limit their biomedical applications relative to traditional synthetic materials such as plastics. PHAs have poor mechanical properties, which restrict their use as film packaging material and vascular or controlled drug delivery systems.⁸⁵ Other disadvantages include high production cost, incompatibility with conventional thermal processing techniques, limited functionalities and susceptibility to thermal degradation. Many strategies have been employed to improve the properties of PHAs, including blending with natural raw materials or other biodegradable polymers and chemical modification. Two common methods of chemical modification are block

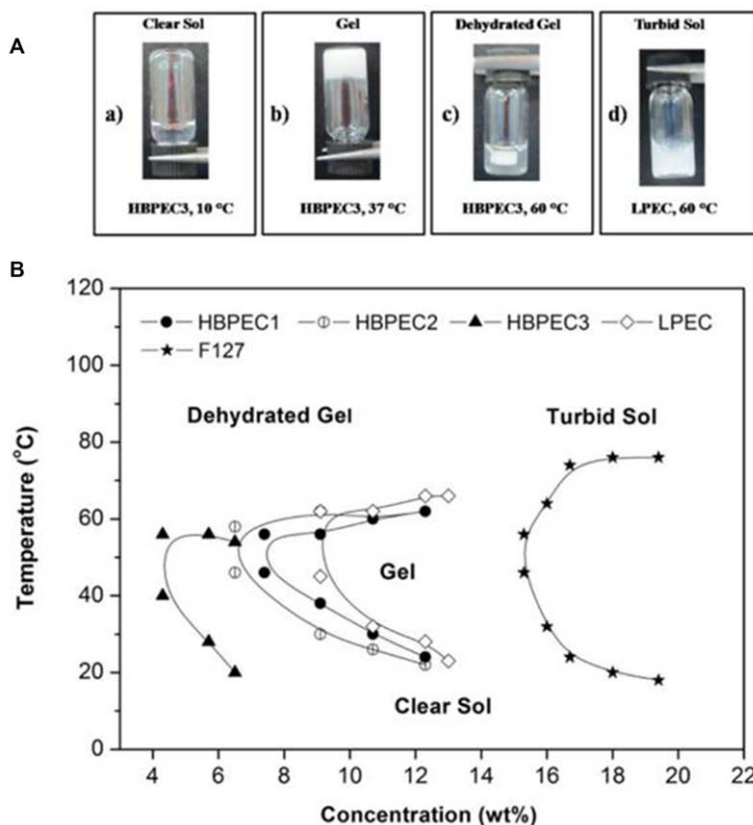


Figure 3.10 (A) Graphics showing the gel transition of HBPEC3 (5.7 wt% in H₂O) with increasing temperature. (B) Sol-to-gel phase diagrams of HBPEC copolymers in comparison with LPEC and Pluronic F127 copolymers.⁷⁷ Reprinted with permission from ref. 77. Copyright 2012 American Chemical Society.

copolymerization and graft copolymerization. PHB is also highly crystalline and hydrophobic, showing greater hydrophobicity than PLA and PCL. Therefore, Loh and co-workers copolymerized hydrophobic and biodegradable PHB blocks with hydrophilic PEG and temperature-sensitive PPG segments to increase the resilience of the copolymer-based hydrogels by formation of extra physical cross-links.⁸⁶ A series of new biodegradable multi-block amphiphilic and thermo-sensitive poly(PEG/PPG/PHB urethane) materials were synthesized by a simple synthetic procedure (Figure 3.12). The water-soluble copolymers obtained had better thermal stability than the PHB precursors, and very low CMC. Their aqueous solutions underwent a reversible sol-to-gel transition with low CGC, ranging from 2 to 5 wt% (Figure 3.13).

Loh, Guillaume and co-workers also reported novel thermogels based on multi-arm PHB-based triblock copolymers, PHB-*b*-PNIPAAm-*b*-(PPEGMEMA-*co*-PPPGMA) (Figure 3.14).⁸⁷ PHBs with different arms and different

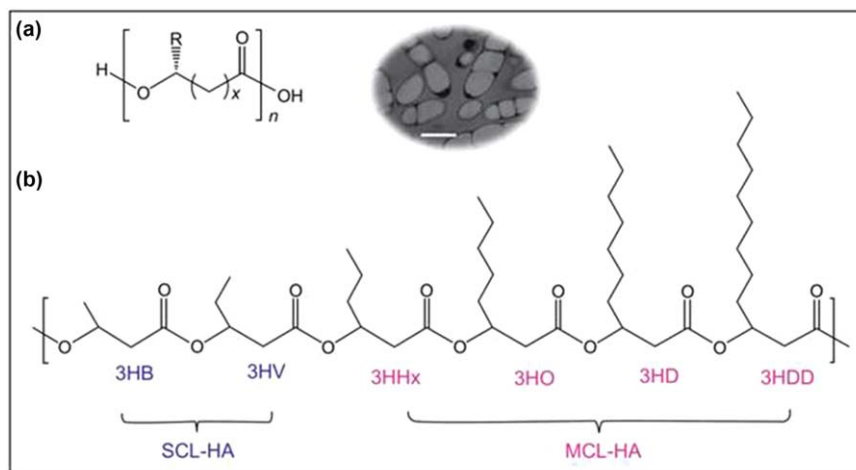


Figure 3.11 (a) General molecular formula of PHAs. (b) Examples of common PHAs. Inserted TEM image that shows thin sections of recombinant *R. eutropha* PHB – 4 cells that contain large amounts of P(3HB-co-5mol% 3HHx). The bar represents 0.5 μm.⁸⁴ Reproduced from ref. 84 under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>.

numbers of the hydroxyl end-capping groups were prepared by transesterification using different ‘cutting agents’, such as hexanol, ethylene glycol and erythritol. Triblock copolymers were then synthesized from bromoesterified PHB by ATRP of PNIPAAm, followed by PEGMEMA and PPGMA. The resulting copolymers gained biodegradability and thermo-responsiveness. These copolymers featured different architectures and distinct hydrophilic/hydrophobic regions and so displayed different gelation behaviour. One-arm PHB-based linear copolymers can only form micellar solutions, while multi-arm PHB-based star-shaped copolymers can form gels with enhanced, solid-like mechanical properties. Cytotoxicity assays and doxorubicin (DOX) release tests indicated that these copolymers were biocompatible and promising candidates for components of drug-delivery systems.

Recently, a modified hydrophobic poly[(*R*)-3-hydroxybutyrate-co-4-hydroxybutyrate] (P3HB4HB) block was copolymerized with PEG and PPG blocks to provide the thermo-responsive multiblock copolymers poly(PEG/PPG/P3HB4HB urethane)⁸⁸ (Figure 3.15). Aqueous solutions of copolymers containing P3HB4HB segments showed lower CMC and CGC values (ranging from 2 to 6 wt%) compared with those containing only P3HB. The images of the sol-to-gel-to-turbid solution transition with increasing temperature and the illustration of the gel formation stages are shown in Figure 3.16. Thermogels containing P3HB4HB degraded more slowly and exhibited relatively faster release of DOX compared with P3HB-containing thermogels.

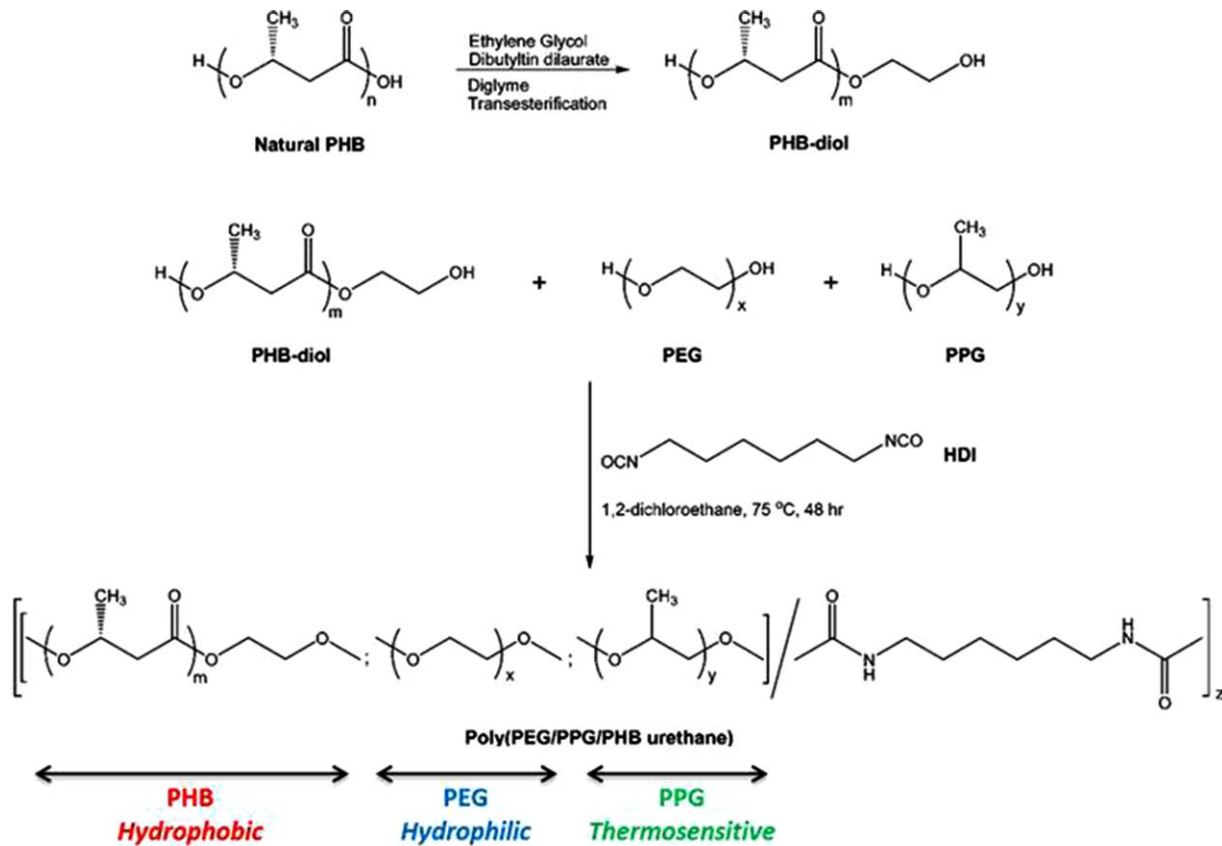


Figure 3.12 Synthetic routes to PHB-diol and Poly(PEG/PPG/PHB urethane).⁸⁶
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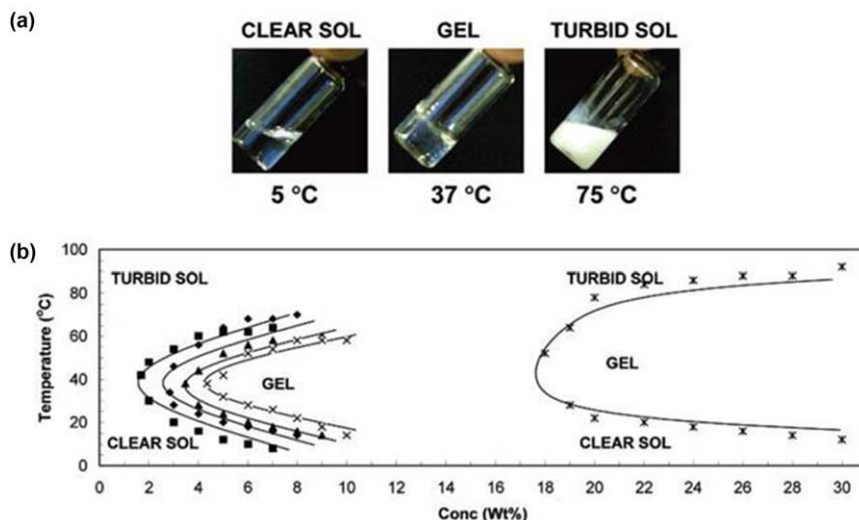


Figure 3.13 (a) Graphics showing the gel transition of aqueous solutions of poly(PHB/PEG/PEG urethane) with increasing temperature. (b) Sol-to-gel phase diagrams of poly(PEG/PPG/PHB urethane) in aqueous solutions (left), in comparison to EG₁₀₀PG₆₅EG₁₀₀ triblock polymer (right).⁸⁶ Reprinted with permission from ref. 86. Copyright 2007 American Chemical Society.



Figure 3.14 Schematic drawing of the proposed micellar network structures of gels G1, G3, and G9.⁸⁷ Reproduced from ref. 87 with permission from John Wiley and Sons, © 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

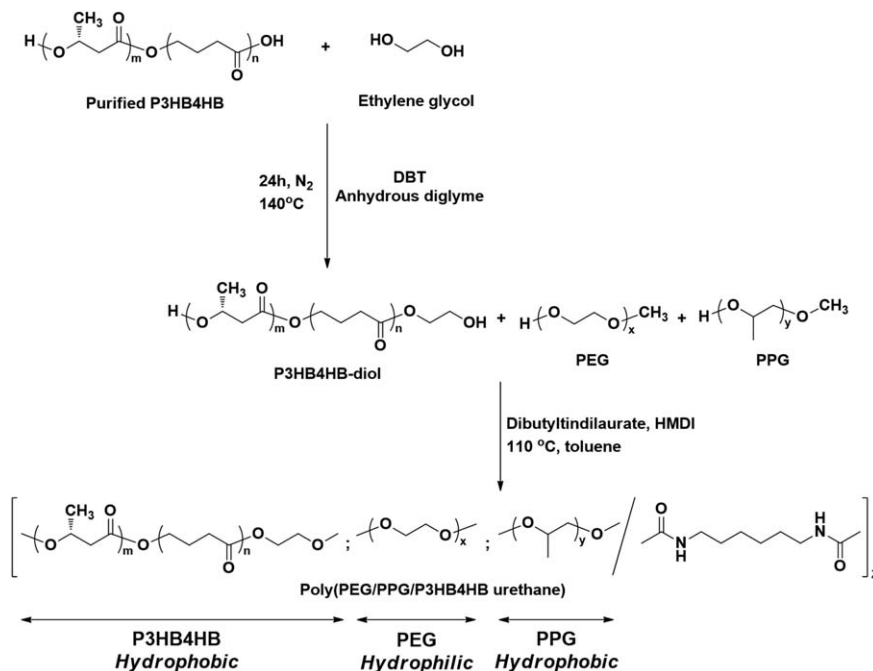


Figure 3.15 Synthetic routes to P3HB4HB-diol and poly(PEG/PPG/P3HB4HB urethane)s.⁸⁸ Reproduced from ref. 88 with permission from John Wiley and Sons, © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.2.4 Poly(glycerol sebacate)-based Thermogelling Systems

Poly(glycerol sebacate) (PGS) was first synthesized using a simple polycondensation reaction of glycerol and sebacic acid by Wang and co-workers in 2002.⁸⁹ Both starting materials are non-toxic, which gives PGS superior biocompatibility. Glycerol is colourless and odourless, and is an FDA-approved food additive, which has been widely used in the pharmaceutical and food industries. It is water-soluble and hygroscopic because of its three hydroxyl groups and low molecular weight. The other starting material, sebacic acid, is also a non-toxic acid and approved by the FDA for medical applications. PGS is a hydrophobic and elastomeric polymer with alternating units. It undergoes controlled and linear degradation compared with the drastic degradation of conventional degradable materials, such as PLA and PGA. The modulus of fully cured PGS is around 0.28 MPa, which is near to the values of some human tissue. However, PGS also has its limitations, such as high hydrophobicity, low water uptake and harsh synthetic conditions. To overcome these restrictions, a range of methods, including blending and chemical modification, have been developed. PGS has attracted increasing attention for biomedical applications such as drug delivery, biocompatible coatings on implants and tissue engineering because of its soft, robust and flexible nature,

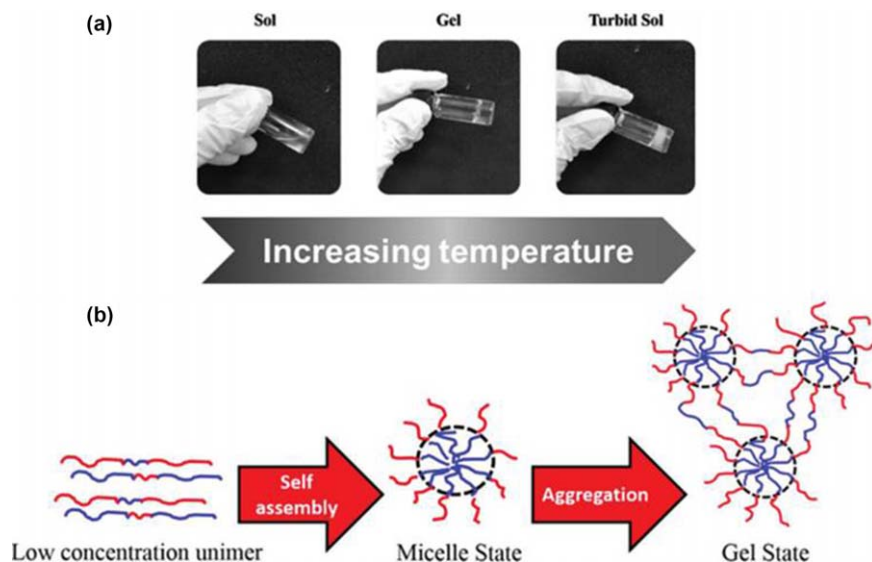


Figure 3.16 (a) Sol-to-gel-to-turbid solution transition upon temperature change. (b) Illustration of gel formation stages. The copolymers containing hydrophobic P3HB4HB and PPG components, and the hydrophilic PEG component is in the unimer form at low concentration; with an increase of the concentration of the copolymer solution, the copolymers will form the micelle by self assembly, and further form the gel state by aggregation. Reproduced from ref. 88 with permission from John Wiley and Sons, © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

and relatively low cost and non-toxicity. Recent modifications of PGS have significantly expanded its biomedical application opportunities.⁹⁰

In 2015, Ye and Loh successfully synthesized a PGS macroinitiator, which enabled the synthesis of PGS-based copolymers with a wide range of monomers, including methacrylates, styrenes and methacrylamide using ATRP.⁹¹ Polyethylene glycol methyl ether methacrylate (PEGMEMA) blocks were chosen to demonstrate the feasibility of this macroinitiator (Figure 3.17). A PGS-PEGMEMA/ α -CD supramolecular hydrogel system was obtained by mixing copolymer PGS-PEGMEMA and alpha-cyclodextrin (α -CD). The PGS-PEGMEMA copolymer has a brush-like structure, and the α -CD molecules threaded onto each PEG segment to form columns of inclusion complexes. These brush-like columns of PEGMEMA/ α -CD inclusion complexes interact with each other *via* hydrogen bonding to form the hydrogel matrix that was able to hold water (Figure 3.18a). This hydrogel showed rapid gelation, low CGC ($\sim 5.2\%$), low and tunable upper critical solution temperature (UCST) ($< 90^\circ\text{C}$, Figure 3.18b) and rapid self-healing ability with a relatively high modulus (~ 100 kPa) comparable to that of human soft tissue. It was also strong enough for injection and is therefore suitable for use in injectable sustained-release drug delivery, cell delivery and tissue engineering scaffold applications.

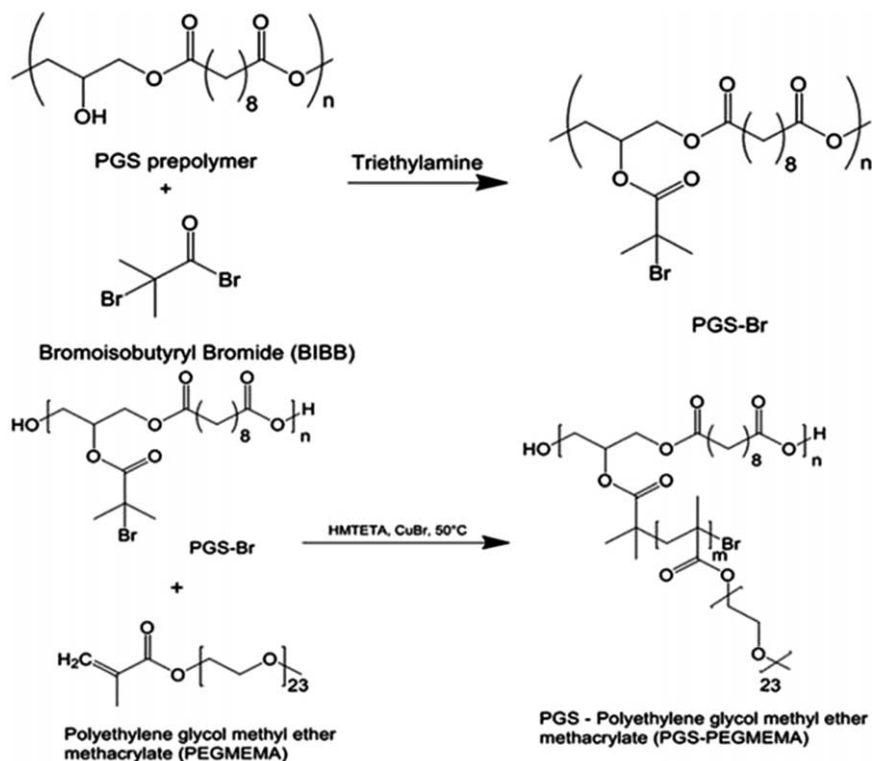


Figure 3.17 The synthetic route to PGS-Br macroinitiator by bromination, and to PGS-PEGMEMA by ATRP.⁹¹ Reproduced from ref. 91 with permission from The Royal Society of Chemistry.

In the following year, the same research group demonstrated self-healing of this hydrogel system, which also proved to be biocompatible and biodegradable, and suitable for sustained release of chemotherapeutic drug (DOX) without the initial burst effect. *In vitro* experiments indicated linear mass erosion and a biphasic drug release profile. During this erosion, the un-eroded portion was still able to maintain its integrity and remained as a gel (Figure 3.19). Hence, the desired release rate could be calculated and manipulated with the correct hydrogel formulation.⁹²

In 2015, Chen and co-workers synthesized a series of PGS-PEG copolymers with varying molecular weight of PEG blocks by a facile, solvent-based two-step method (Figure 3.20).⁹³ These polyester-based polyurethane (PEU) hydrogels were chemically cross-linked, structurally stable, thermo-responsive, stretchable, biodegradable and biocompatible. They showed reversible responses to temperature change (5–37 °C), with the swelling ratio at equilibrium varying from 499% to 12%. They also demonstrated good stretchability and full shape recovery after compression, with similar mechanical properties to adipose tissues. The results of *in vitro* cell tests,

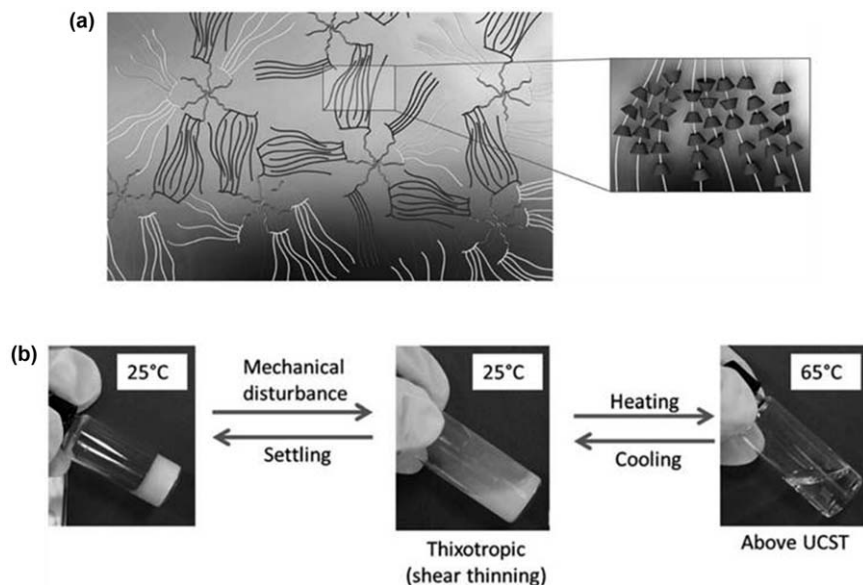


Figure 3.18 (a) Schematic illustration of a PGS-PEGMEMA/ α -CD hydrogel system; (b) Photographs of 2% PGS-PEGMEMA1 + 5% α -CD gel demonstrating shear thinning and exhibiting the characteristics of a UCST system.⁹¹ Reproduced from ref. 91 with permission from The Royal Society of Chemistry.

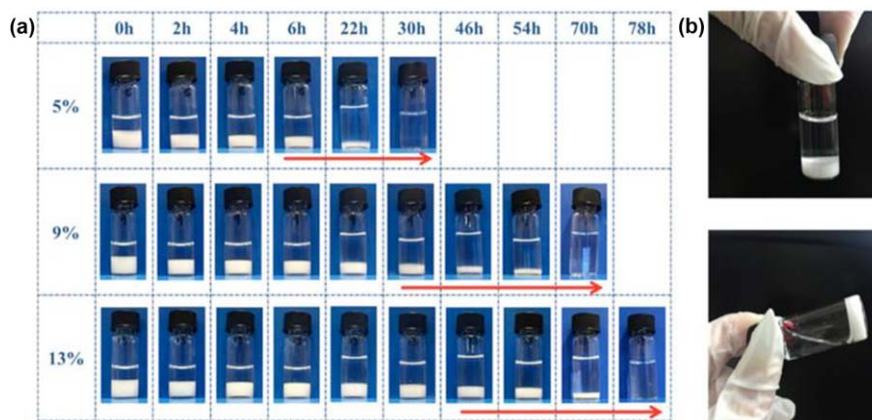


Figure 3.19 Images of the PGS-PEGMEMA1/ α -CD hydrogel system. (a) Images taken at various times for 5%, 9% and 13% α -CD. The hydrogels went through two phases of erosion at all α -CD concentrations. During phase I there was no visible change to the gel, and in phase II where there was a gradual one-dimensional erosion from the top surface. Phase II is marked by the arrow. (b) Image taken at 54 h for α -CD concentration of 13%. The hydrogel had started to erode but the un-eroded portion was able to maintain its integrity and remain as a gel.⁹² Reproduced from ref. 92 under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>, © 2016 by the authors.

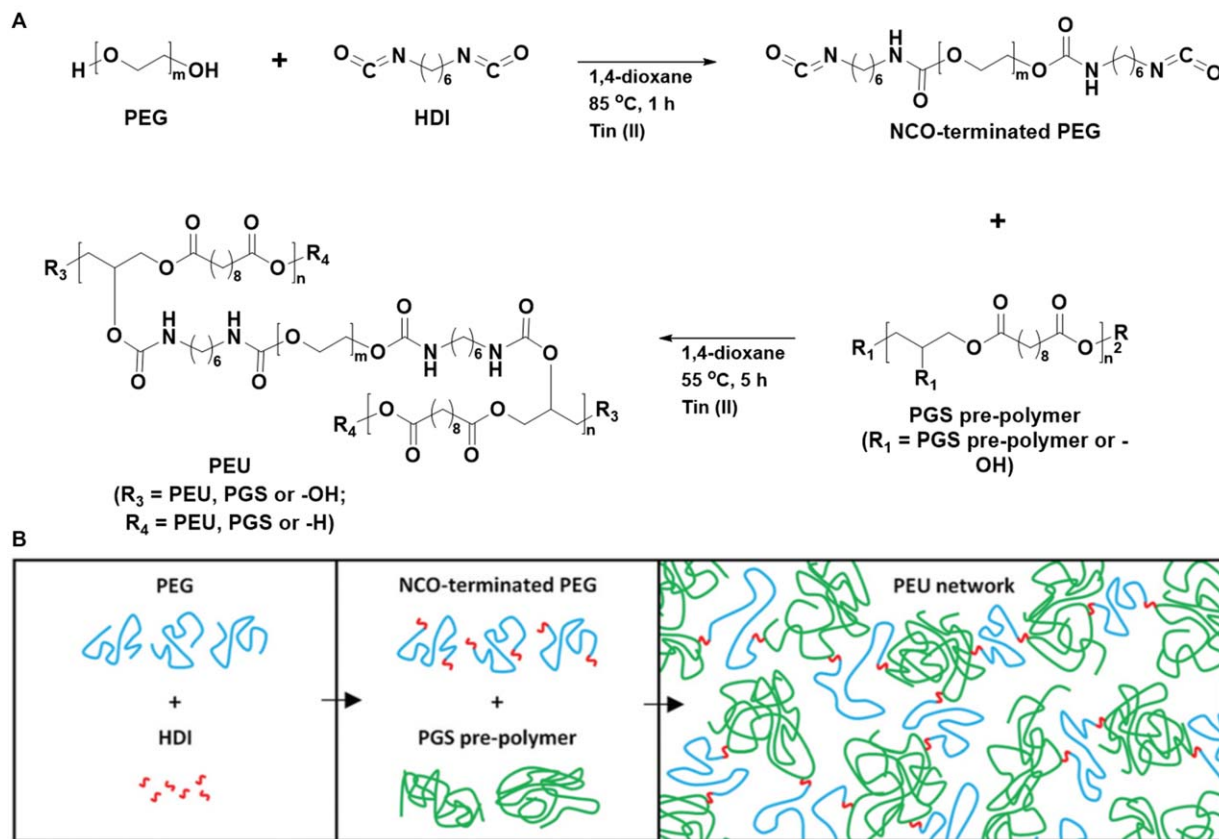


Figure 3.20 (A) Synthetic route to PEUs. (B) Sketch showing the formation of the PEU network.⁹³
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drug delivery, thermal actuation and ultralow power generation tests demonstrated the versatility of these PEU hydrogels for a variety of biomedical and engineering applications.

3.3 Application of Polyester-based Thermogelling Systems

3.3.1 Therapeutic Delivery

The challenge of developing sustained release drug-delivery systems is finding the balance between maintaining the optimum drug concentration in the blood for the desired therapeutic effect and limiting the critical value of concentration to reduce toxicity after repeat dosage.^{94,95} Biodegradable polyester-based amphiphilic block copolymers provide a way to release drugs at a controlled rate through thermo-responsive hydrogel formation and slow degradation. The amphiphilic nature of the block copolymers is the reason they self-assemble into micelle-like structures, which contain a hydrophobic inner core and a hydrophilic surface in selective solvents, and form hydrogels with temperature change. Hence, drugs with a hydrophobic nature can be incorporated into the core in aqueous conditions.⁹⁶ Polyester-based thermogels, including PLA, PCL, PHB and PGS, are excellent candidates for controlled therapeutic delivery systems.

Wu and co-workers employed thermogelling, biocompatible and hydrolytically degradable poly(PEG/PPG/PLA urethane) to conduct drug delivery studies involving the anti-tumour drug PTX *in-vivo*.⁵⁶ Drug release and elimination times were prolonged when compared with conventional PEG-PPG-PEG triblock copolymer-based hydrogels (Figure 3.21). Drug-loaded thermogels could effectively inhibit the growth of tumours (hepatocellular carcinoma) in nude mice with very low toxicity (Figure 3.22).

In 2008, Lee *et al.* synthesized and characterized a series of PCL-based multiblock poly(ester amino urethane)s, denoted as (PCL-PEG-PCL-PAU)_n. They then tested *in vivo* the gel-forming properties of the copolymers in mice, which demonstrated that these gels could be formed *in situ* in a short time (Figure 3.23a). They also investigated the *in-vitro* drug release of these hydrogels using PTX, demonstrating that the release could persist over 1 month under physiological conditions⁷³ (Figure 3.23b). Another PCL-based hydrogel system (PCL-PEG-PCL triblock copolymers) for drug delivery underwent sol-to-gel transition when heated from room temperature (25 °C) to body temperature (37 °C).⁹⁷ The *in vitro* hydrophobic honokiol release results showed a notable difference between rapid release of free honokiol and sustained release of honokiol in hydrogel. Cytotoxicity results were also favourable.

In 2009, a novel kind of PCL-based pentablock copolymer PAE-PCL-PEG-PCL-PAE was chosen for a long-term injectable insulin delivery system.⁷⁶ *In-vivo* hydrogel formation testing was carried out by injecting aqueous

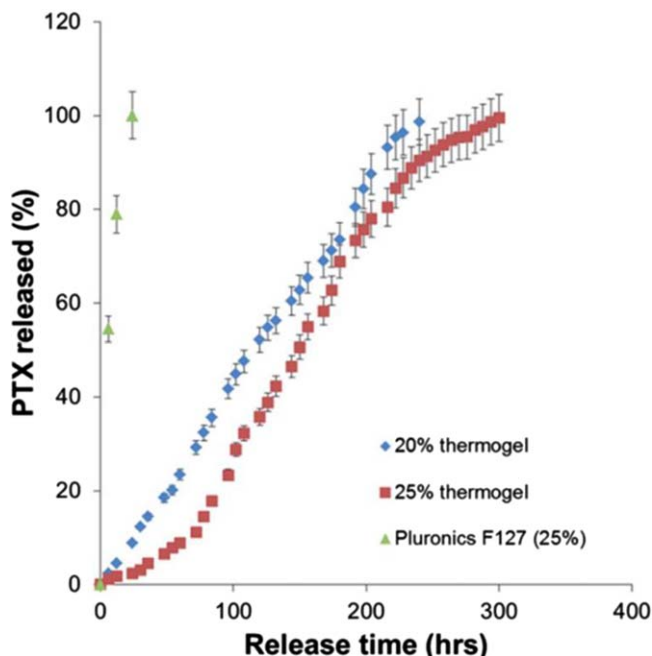


Figure 3.21 PTX release profiles for poly(PEG/PPG/PLA urethane) hydrogels of different copolymer compositions (20 and 25 wt%) in comparison with PEG-PPG-PEG triblock copolymer (Pluronic F127, 25 wt%).⁵⁶ Reproduced from ref. 56 with permission from The Royal Society of Chemistry.

copolymer solutions into rats. Quick hydrogel formation with no inflammation around the hydrogel site was observed. This study showed that insulin release was at a slow and steady rate for 15 days and the insulin level depended on both copolymer concentration and initial insulin loading into the hydrogel. Diabetic rats were treated over 7 days by injecting a complex mixture of 10 mg ml^{-1} insulin in a 30 wt% copolymer solution. The performance of this PAE-PCL-PEG-PCL-PAE copolymer-based insulin delivery system demonstrated its therapeutic potential. Like PCL, poly(ϵ -caprolactone-co-lactide) (PCLA)-based copolymers are also biodegradable with potential for use in sustained drug delivery systems. A pH and temperature dual responsive block copolymer OSM-PCLA-PEG-PCLA-OSM has been synthesized and loaded with Paclitaxel (PTX).^{98,99} The sol-to-gel transition phase diagram of PTX-loaded copolymer solutions shifted to a lower temperature region compared with PTX-unloaded solutions, due to the salting-out effect of PTX. Moreover, copolymer solutions could form gel under low pH conditions, while maintaining the sol state at high pH. *In vitro* release of PTX from hydrogel was in a sustained manner without burst release, regardless of loading amount. Anti-tumour efficacy was observed *in vivo* on subcutaneous injection in tumour-bearing mice.

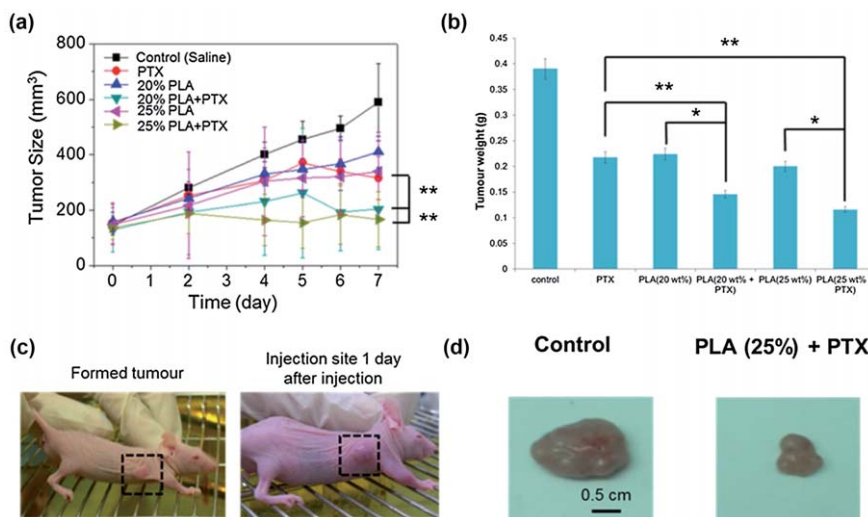


Figure 3.22 (a) Inhibition of tumour volume by intratumoural injection of saline, PTX or PTX-loaded thermogels, dorsal subcutaneous implantation of HepG2 cancer cells into mice was followed by administration of each solution after tumours had reached a volume of $\sim 140 \text{ mm}^3$. (b) Inhibition of tumour weight by intratumoural injection of $100 \mu\text{l}$ of saline, PTX or PTX-loaded PLA gels, dorsal subcutaneous implantation of HepG2 cancer cells into mice was followed by administration of each solution after tumours had reached a volume of $\sim 140 \text{ mm}^3$, the excised tumour removed after 7 days was weighted for evaluation. (c) Induction of tumour growth in mice and the injection site 1 day after injection. (d) The excised tumour removed after 7 days treated with or without PTX treatment, Scale bars represent 0.5 cm * $p < 0.01$ vs. non-drug loaded thermogel; ** $p < 0.001$ vs. direct injection of PTX without thermogel.⁵⁶ Reproduced from ref. 56 with permission from The Royal Society of Chemistry.

PHB-based copolymer thermogels with promising drug release results have also been reported. Poly(PEG/PPG/PHB urethane) copolymers were shown to be thermo-responsive and biodegradable,⁸⁶ and were subsequently investigated for protein release.¹⁰⁰ The release rate could be controlled by varying the composition and concentration of the copolymers. In 2016, these thermogelling copolymers were chosen as drug carriers for the treatment of tumours *in vivo*.¹⁰¹ Drug-loaded thermogels could achieve the controllable release of PTX and DOX by adjusting polymer concentrations. The injected drug-loaded hydrogels effectively inhibited tumour growth *in vivo* using mice model of hepatocarcinoma by intratumour sustained release of the chemotherapeutic agents (Figure 3.24).

Recently, Wee *et al.* also demonstrated drug (DOX) release using novel PEG-PPG-P3HB4HB copolymer based hydrogels.⁸⁸ Although exhibiting an initial burst of DOX, the subsequent release was sustained. P3HB4HB-based hydrogels displayed faster rates of DOX release than P3HB thermogels.

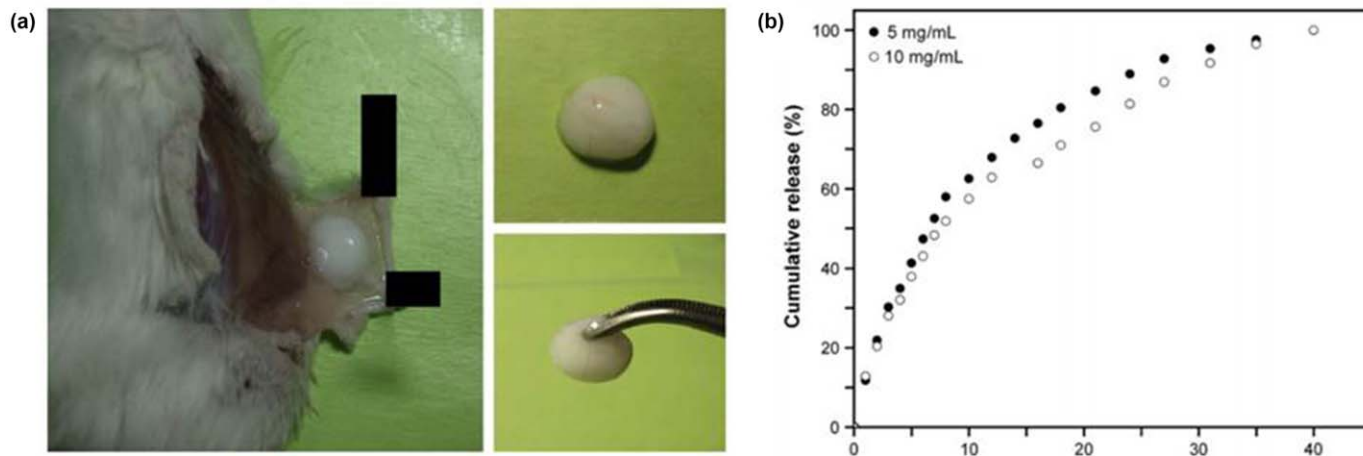


Figure 3.23 (a) *In vivo* gel formation. Photographs were taken 15 min after subcutaneous injection of the 20 wt% multiblock copolymer ((CL₁₉-EG₄₅-CL₁₉-AU₁₀)_{4.1}) solution into the mouse. (b) *In vitro* cumulative release profiles of PTX from the copolymer hydrogel in PBS solution (containing 2.4 wt% Tween 80 and 4 wt% Cremophor EL) at pH 7.4 and 37 °C.⁷³ Reproduced from ref. 73 with permission from the Elsevier, Copyright 2008.

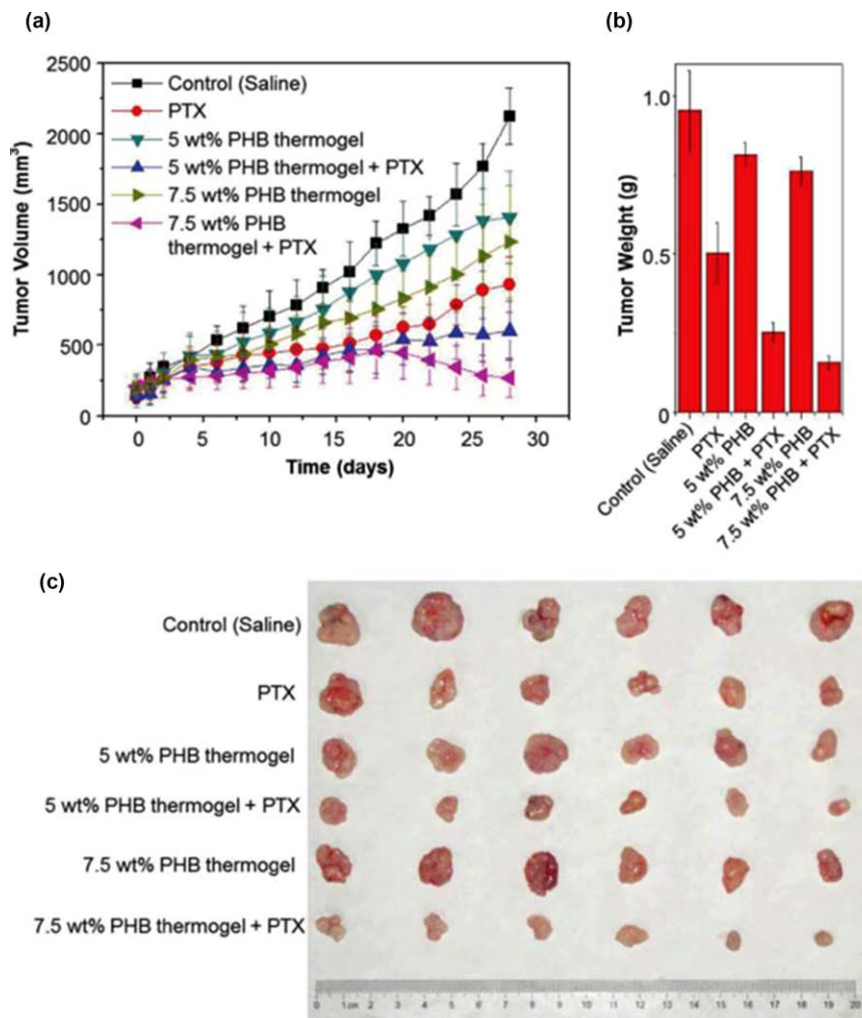


Figure 3.24 (a) Tumour volume growth after intratumoural injection of normal saline, paclitaxel only or paclitaxel/thermogel formulations. Initial dorsal subcutaneous tumour implantation was conducted by injecting HepG2 cancer cells into mice. The experiments started with the tumour size reached $\approx 140 \text{ mm}^3$. Experimental results were displayed as mean with SD error bars ($n=6$). (b) Inhibition of tumour weight after intratumoural injection of normal saline, paclitaxel only or paclitaxel/thermogel formulations. The subcutaneous tumors, excised after 28 days, were weighed and recorded. Experimental results were presented as mean with the SD errors ($n=6$). (c) The images of excised tumour after different formulated treatments for 28 days.¹⁰¹ Reproduced from ref. 101 with permission from John Wiley and Sons, © 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

A self-healing PGS-PEGMEMA/ α -CD hydrogel system demonstrated a bi-phasic erosion profile, but upon increasing the α -CD concentration, the hydrogel was able to reduce the initial burst effect and prolong drug release to over 60–80 hours.⁹²

3.3.2 Tissue Engineering

Hydrogels have been widely studied as scaffolds to guide tissue growth. Hydrogels can promote cell migration, angiogenesis and rapid nutrient diffusion. Various polymeric and hydrogel systems for tissue engineering applications have been reported.^{102–106} Initially, hydrogels based on naturally derived polymers, such as gelatin, collagen and chitosan were investigated. However, poor mechanical properties limited their applications. Therefore, hydrogels based on synthetic polymers with controlled structures and functions were developed with suitable degradation rates, mechanical properties and cell adhesion ability.¹⁰⁶

Narayanan *et al.* have summarized PLA-based biomaterials for regenerative engineering,¹⁰³ describing PLA-based copolymers as the “gold standard” for many regenerative engineering applications because of their easy fabrication, biodegradability and compatibility with biomolecules. In 2002, Jeong *et al.* synthesized PLGA-*g*-PEG copolymers and conducted both sustained injection insulin delivery and tissue engineering testing *in vivo* using animal models.⁶ Articular cartilage defects were manually created in rabbits and treated with autogenic chondrocyte cells suspended in PLGA-*g*-PEG hydrogel solutions. Treatment was successful, with the cut repairing, permitting unhindered weight bearing (Figure 3.25).

A novel eight-arm star-shaped PEG-*b*-PLLA-cholesterol was synthesized as a biodegradable copolymer derivative by Nagahama and co-workers.¹⁰⁷ An aqueous solution of this material underwent sol-to-gel transition at 34.8 °C, but only after cholesterol was introduced. Driven by the self-assembly of cholesterol groups, an extracellular matrix-like micrometer-scale network structure was created. This network structure contained favourable porosity for 3D proliferation of cells inside the hydrogel, and good cell proliferation of encapsulated L929 cells was achieved *in vitro*.

PCL-based copolymer hydrogels have also been reported to have potential for use in tissue engineering. A series of hyperbranched and biodegradable amphiphilic polyurethane block copolymers PCL-PEG-Glycerol (CEG) were synthesized and characterized by Li and co-workers.¹⁵ Porous morphology and tunable rheological properties made them suitable for 3D living cell encapsulation and delivery. The encapsulated cells preserved good cell viability and good proliferation after recovery from the hydrogel. These promising results indicated the potential for hydrogels as injectable scaffolds. In another example, thermo-sensitive MPEG-PCL diblock copolymer-based hydrogels have been successfully used in bone tissue engineering.¹⁰⁸ Copolymer solutions containing rat bone marrow stromal cells (rBMSCs) and dexamethasone were injected into rats, forming gel scaffolds *in situ* at

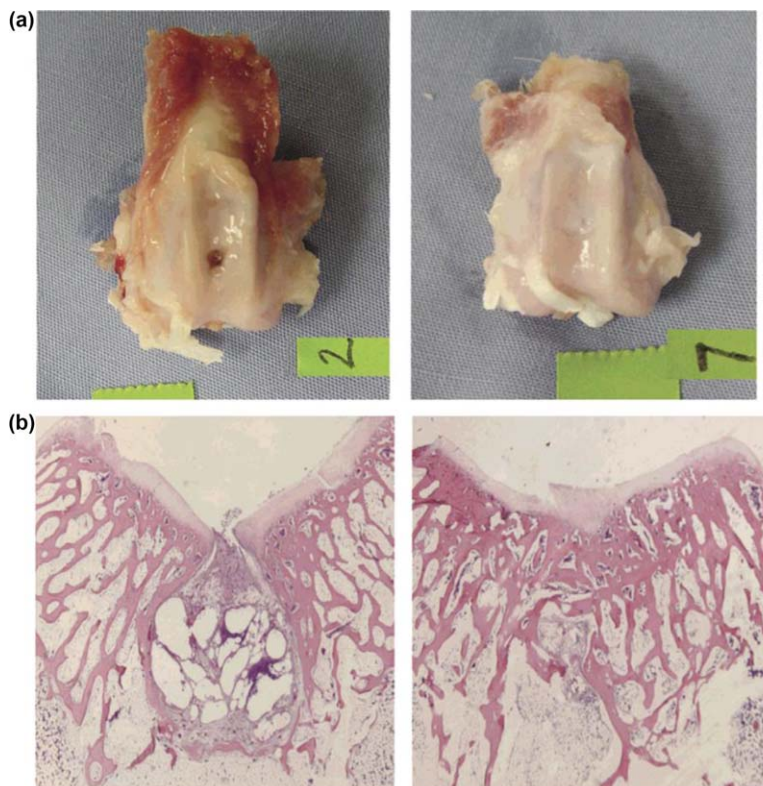


Figure 3.25 Cartilage repair: (a) overview of control sample using poly(*N*-isopropyl acrylamide-*co*-acrylic acid) (left) and PLGA-*g*-PEG (right); (b) histology of control sample using poly(*N*-isopropyl acrylamide-*co*-acrylic acid) (left) and PLGA-*g*-PEG (right).⁶ Reprinted with permission from ref. 6. Copyright 2002 American Chemical Society.

injection sites and viable bone formation over 4 weeks. Biocompatible MPEG-PCL copolymer-based gel implants could maintain good mechanical properties and were sterilizable to prevent infection. A novel pH/thermo-sensitive and biocompatible block copolymer OSM-PCLA-PEG-PCLA-OSM has been developed and used as a scaffold for autologous bone tissue engineering.¹⁰⁹ Sol-to-gel transition of copolymer solution was observed at 37.8 °C when the pH was changed from 8 to 7.8, making it an injectable gel. This material was able to encapsulate human mesenchymal stem cells (hMSCs) and recombinant human bone morphogenetic protein-2 (rhBMP-2) with high efficiencies (90% and 85%, respectively). An *in vivo* experiment involved injecting a polymer solution containing hMSCs and rhBMP-2 into the back of mice. Over a 7-week period, mineralized tissue formation and high levels of alkaline phosphatase activity in the mineralized tissue were observed. These results demonstrated that this dual responsive hydrogel was

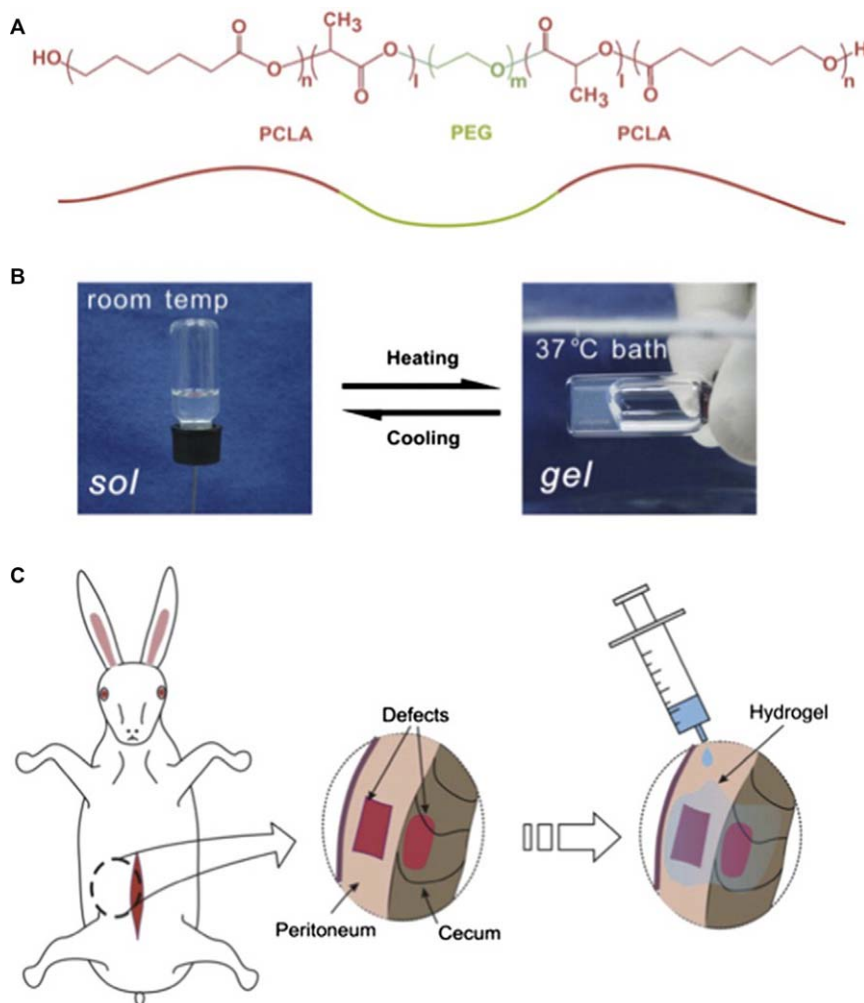


Figure 3.26 (A) Chemical structure of PCLA-PEG-PCLA copolymer. (B) Photographs of the polymer solution (20 wt% in normal saline solution) as a sol at room temperature and a gel after heated to body temperature. (C) Insertion of PCLA-PEG-PCLA hydrogel onto the peritoneal wall defect of a rabbit. The defect ($4 \times 3 \text{ cm}^2$) comprising the parietal peritoneum and a layer of muscle ($\sim 1 \text{ mm}$ thick) was excised starting 1 cm from the midline of the peritoneal wall, and the corresponding site on the cecum was abraded until bleeding with a surgical brush.¹¹⁰ Reproduced from ref. 110 with permission from Elsevier, Copyright 2011.

able to act as an injectable scaffold for bone tissue engineering. Moreover, the morphological characteristics of the hydrogel could be maintained for 7 weeks and the size shrank due to degradation from hydrolysis of polyester blocks.

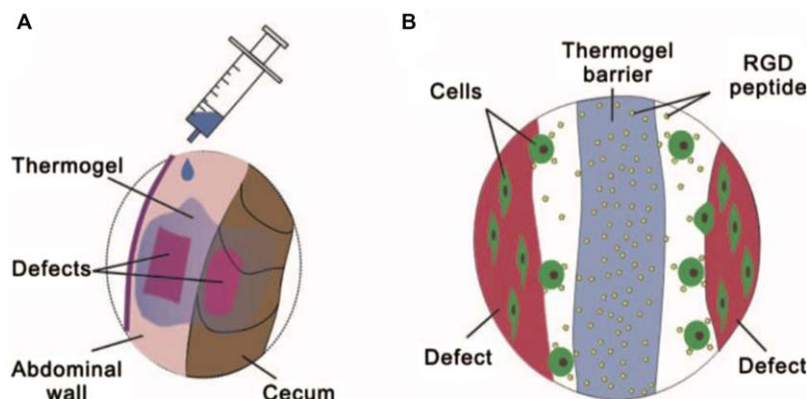


Figure 3.27 Application of an RGD-loaded hydrogel to prevent post-operative peritoneal adhesions. (A) A sol from mixing a PCL-PEG-PCL aqueous solution with free RGD peptides is injected to cover the defects generated in the abdominal wall and cecum of rabbits. The sol becomes a thermogel at the body temperature. (B) The hydrogel acts as a physical barrier between the abdominal wall and cecum, and releases RGD peptides to assist anti-adhesion between tissues.¹¹¹ Reproduced from ref. 111 with permission from John Wiley and Sons, Copyright © 2012 Wiley Periodicals, Inc.

Recently, a novel biodegradable triblock copolymer PCL-PEG-PCL-based thermogel was reported to serve as a barrier material to prevent adhesion after surgery (Figure 3.26).¹¹⁰ *In vivo* anti-adhesion studies on rabbits indicated its effectiveness in preventing post-operative adhesion. Peptides containing the sequence arginine-glycine-aspartate (RGD) have also been demonstrated to prevent post-operation adhesion between cells and surfaces of barrier devices. The same group did further study to investigate the consequences of encapsulated RGD peptides in PCL-PEG-PCL thermogel.¹¹¹ Long-term release of RGD from the hydrogel was observed over 7 days using a rabbit model with significant reduction of post-operation adhesion. Better efficiency was obtained with the combined RGD-encapsulated hydrogel than for either individual component (Figure 3.27).

3.4 Conclusion

In summary, polyester-based thermogelling systems have the advantages of polyester biodegradation, biocompatibility and synthetic versatility. Controlled therapeutic delivery has proven effective in numerous cases studied, such as anti-tumour treatment in rat models. This material is being increasingly used in tissue engineering as an injectable scaffold for cell growth. Polyester-based thermogelling systems will undoubtedly have a bright future in the healthcare industry.

Abbreviations

3D	three dimensional
PEO	poly(ethylene oxide)
PPO	poly(propylene oxide)
CMC	critical micelle concentration
CMT	critical micelle temperature
PNIPAAm	Poly(<i>N</i> -isopropyl acrylamide)
PLA	poly(lactic acid)
PLLA	poly(L-lactic acid)
PDLA	poly(D-lactic acid)
PDLLA	poly(D,L-lactic acid)
PEG	poly(ethylene glycol)
NMR	Nuclear magnetic resonance spectroscopy
DLS	Dynamic light scattering
PPG	poly(propylene glycol)
HMDI/HDI	hexamethylene diisocyanate
CGC	Critical gelation concentration
LCST	lower critical solution temperature
PTX	paclitaxel
PCL	Polycaprolactone
FDA	US Food and Drug Administration
PGA	Polyglycolic acid
PAU	Poly(amino urethane)
HEP	1,4-Bis(hydroxyl ethyl)piperazine
ATRP	Atom transfer radical polymerization
PAE	Poly(β -amino ester)
HBPEC	hyperbranched poly(PPG/PEG/PCL urethane)s
LPEC	linear poly(PPG/PEG/PCL urethane)s
P3HB, PHB	Poly(3-hydroxybutyrate)
PHAs	Polyhydroxyalkanoates
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
3HHx	3-hydroxyheanoate
3HO	3-hydroxyoctanoate
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
PPEGMEMA	poly(methyl ether methacrylate)- <i>g</i> -poly(ethylene glycol)
PPPGMA	poly(methacrylate)- <i>g</i> -poly(propylene glycol)
DOX	doxorubicin
P3HB4HB	poly[(<i>R</i>)-3-hydroxybutyrate- <i>co</i> -4-hydroxybutyrate]
PGS	Poly(glycerol sebacate)
α -CD	Alpha-cyclodextrin
PEGMEMA	Polyethylene glycol methyl ether methacrylate
UCST	Upper critical solution temperature
PEU	Polyester-based polyurethane

PCLA	Poly(ϵ -caprolactone-co-lactide)
OSM	Oligomeric sulfamethazine
PLGA	Poly(lactic-co-glycolic acid)
MPEG	Methoxy poly(ethylene glycol)
CEG	PCL-PEG-Glycerol
hMSCs	Human mesenchymal stem cells
rhBMP-2	Recombinant human bone morphogenetic protein-2
RGD peptide	Arginine-glycine-aspartate peptide

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CHAPTER 4

Biodegradable Thermogelling Polymers for Drug Delivery

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4.1 Introduction

Drug delivery can be defined as an approach to transport pharmaceutical compounds in the body to achieve desired therapeutic effects. This is often carried out using a drug-transporting carrier or agent and various routes of administration into the body, such as through the mouth (peroral), through the skin (topical), through the trans-mucosal exposed membranes, by inhalation, injection and nanoneedle arrays. The carriers, which can include liposomes, polymeric micelles, microspheres, protein complexes or conjugates and even erythrocytes, need to be safely circulated in the body to reach the target therapy site.

A thermogel is a hydrogel which responds to a change in temperature, typically around the range of body temperature. Thermo-sensitivity is a unique feature of thermogels. At 25 °C, a thermogel may be a liquid, called a

sol, and at 37 °C (body temperature), it undergoes a reversible sol-to-gel transition to form a solid gel. The molecules for therapy such as peptides or drugs are mixed with the polymer solution in a sol state and then the sol is injected at room temperature, which proves to be a minimally invasive drug-delivery method. The drug is thus trapped in the network structure of the gel.¹ Biodegradability means that the thermogel can be degraded in the body hydrolytically or enzymatically, without any external degrading agents. An important aspect of biodegradability is the non-toxic degradation product mixture that results in a buffer system at near neutral pH, which can be metabolized and excreted out of the body as, for example, amino acids, ethanol, phosphates, and ammonia. Examples of common/commercial biodegradable thermogelling polymers are: Pluronic-based triblock copolymers (PEG-X-PEG being most common form due to their hydrophilicity and biocompatibility; ABA or BAB type), chitosan-based copolymers,² hyaluronic acid,³ chitosan⁴ and poly(*N*-isopropylacrylamide)-based⁴ copolymers. These thermosensitive copolymers have been grafted with a variety of biocompatible and biodegradable components containing hydrolysable backbones or easily oxidized side groups, such as poly(*D,L*-lactic acid-*co*-glycolic acid)⁵ (PLGA), poly(*L*-lactic acid)⁶ (PLLA), poly(ϵ -caprolactone)^{7,8} (PCL) or polycaprolactone diol,⁹ poly([*R*]-3-hydroxybutyrate)^{10,11} (PHB), poly(organophosphazene),¹² poly(propylene phosphate),¹³ polyacetal¹⁴ and poly(*ortho* ester).¹⁵ Polypeptides with unique biodegradability, thermosensitivity due to self-assembled secondary or tertiary structures and ionic side groups for customizability, such as poly(*L*-alanine)¹⁶ and poly(*L*-alanine-*co*-*L*-phenyl alanine),¹⁷ are also excellent alternatives. In this review, the most recent developments of biodegradable thermogelling polymers in drug delivery will be highlighted. The focus here is on how they can help in the controlled release of drugs and achieving certain clinical goals, especially in the treatment of diabetes.

4.2 Thermogelling Mechanism

Most of the thermogelling systems used nowadays are triblock copolymers. Triblock copolymers possess the unique property of amphiphilicity due to different moieties, or segments, in the chain. The interactions between different segments of the copolymer chain lead to the formation of micelles under certain conditions. This micelle formation is characterized by the critical micellar temperature (CMT) and critical micellar concentration (CMC).¹⁸ At certain points above CMT and CMC, spherical micelles will be formed because of the inter-chain and intra-chain interactions. Gelation occurs when the micelles pack closely together. Taking Poloxamer 407 (copolymer of ethylene oxide (EO) and propylene oxide (PO)) as an example, the spherical micelles consist of a hydrophobic PO core and hydrophilic EO chains surrounding the core. The gelation mechanism is shown in Figure 4.1.¹⁹ A hydrophobic dye dissolution test can verify the formation of polymeric micelles in aqueous solution.

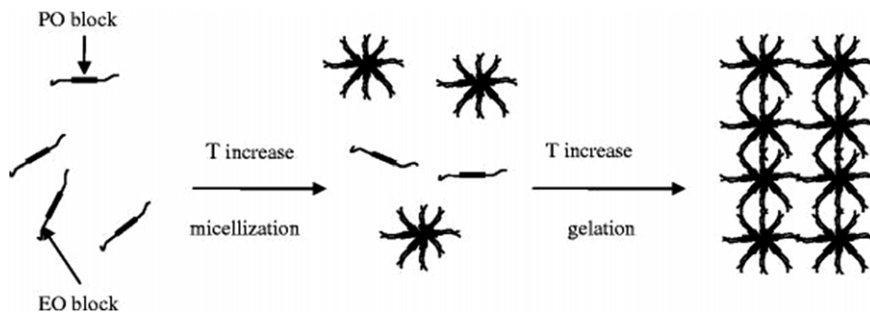


Figure 4.1 Gelling mechanism of Poloxamer 407, a copolymer of ethylene oxide (EO) and propylene oxide (PO). Reprinted by permission from Springer Nature: ref. 19, © Springer Science+Business Media, Inc. (2006).

The chains of a Pluronic-ABA-type polymer consist of EO blocks (portions on both sides) and PO block (middle portion). In a liquid state, the polymer chains are separated by aqueous solvent molecules, and this state is called a sol. As the temperature increases, the hydrophobic PO blocks start to aggregate by hydrophobic interactions forming micelles, with the hydrophilic EO blocks sticking outwards facing the aqueous solvent molecules. Above a certain temperature (called the sol-to-gel transition temperature, or gelation temperature), the micelles start to pack closely together to form a 3D percolated network (a gel), whereby the entanglements of the hydrophilic EO blocks (with hydrogen bonding) serve as physical crosslinks. At a temperature which is significantly above the gelation temperature, the whole gel is no longer able to hold water and precipitates out because the structure becomes too dense.

The gelation temperature can be quite precisely controlled by tuning the composition of the sol by, for example, varying the length of the hydrophilic and hydrophobic segments (changing the ratio of hydrophilic to hydrophobic segments), or using a mixture of copolymers of different states, such as a sol and a precipitate, at the desired temperature, or by using additives such as salts to change the rheological behavior of the sol.

4.3 Mechanism of Drug Release in Thermogels

The release mechanism is complex and depends on many factors. There are two main mechanisms of drug release from hydrogels, namely diffusion and erosion. Other mechanisms include swelling-controlled and chemical release (action by enzymes, proteins, hydrolysis).

The diffusion of a drug out of a hydrogel depends on the chemical composition and concentration of the drug itself (according to Fick's law), as well as the properties of the gel matrix, such as the size of the gel used, mesh size, pore size, crosslinking density, degradability of the gel matrix, the flexibility

of the polymer chains in the matrix and presence of specific interactions between the drug and gel – all of which affect the diffusion coefficient or diffusivity of the drug molecules. Typical mesh sizes reported for hydrogels used for biomedical applications range from 5 to 100 nm in their swollen state, which is larger than most types of small molecule drugs. Therefore, the diffusion of these drugs can only be controlled by factors other than the mesh size. Hydrophilic macromolecules such as oligonucleotides, peptides and proteins have a hydrodynamic radii that matches the range of mesh sizes and possess, and therefore a more sustained release profile.²⁰ Cross-linking density, defined as the average molecular weight (MW) between crosslinks, is affected by the concentration of the polymer used. Higher concentrations result in higher crosslinking density, leading to a reduction in drug mobility in the thermogel matrix.

When a hydrophilic drug is mixed with the sol, the drug molecules will hydrogen bond to the hydrophilic “tails” or entanglements and be trapped in those regions with free volume in the hydrogel matrix. The release of these drugs is mainly by diffusion because they are bound less tightly and near to the periphery (Figure 4.2a). In contrast, a hydrophobic drug molecule will enter the “core” regions of the hydrogel matrix and be trapped within the center of the micelle. Therefore, to release these drugs, some erosion must occur to separate the polymer chains forming the micelles before the interactions with the drug molecules can be overcome. This erosion often

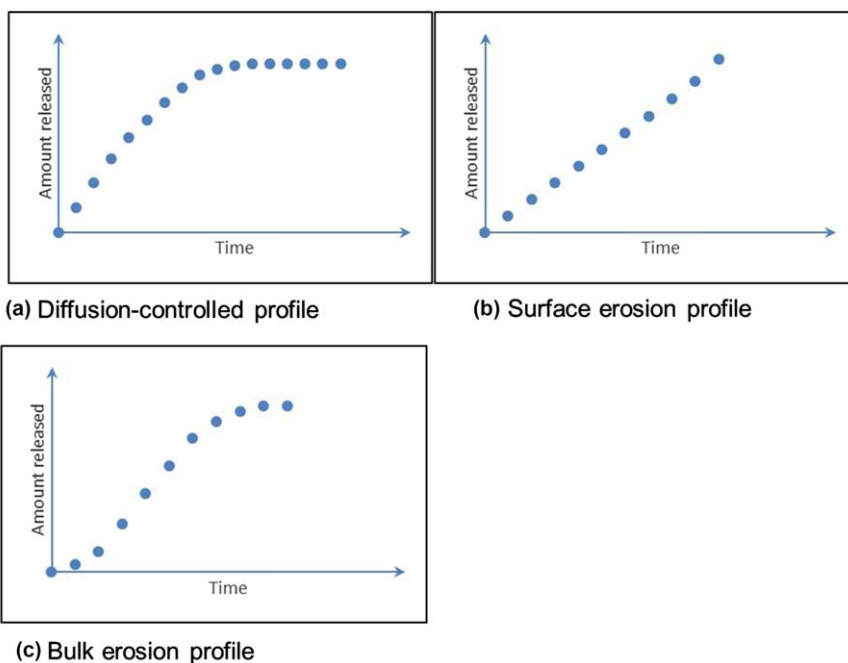


Figure 4.2 Typical drug release profiles from a thermogel.

occurs on the surface of the thermogel, where micelles can break apart (the local concentration on the surface is often lower than the critical micelle concentration) (Figure 4.2b).

For a biodegradable thermogel, the drug release usually involves diffusion and erosion at the same time. Erosion can be of two types, namely bulk erosion and surface erosion. Bulk erosion occurs when the gel is “tight”, *i.e.* water cannot penetrate easily into the gel. This applies to gels with high crosslink density or high hydrophilicity, such as those with poly(lactide-glycolide) components, or drugs with small molecular size (Figure 4.2c). To reduce the initial burst release problem, fast gelation is crucial. After gelation, release of the drug by surface erosion is the preferred mechanism because the release profile is smooth. Surface erosion is prevalent in thermogels with highly hydrophobic groups, such as PEG-PHB-PEG and PEG-PCL-PEG thermogels.²¹

4.4 Advantages and Disadvantages of Thermogelling Polymeric Materials Compared to Other Drug-delivery Methods

- **Comparing invasiveness with other methods.** The gel can be injected subcutaneously or intravenously as a liquid sol prior to the thermogelling process. Compared with oral delivery, injection is a much faster route of administration and results in better adsorption of the drug.
- **Sustained release.** Once injected, the thermogel serves as a depot, releasing the drug in an extended period and in consistent amounts. The conventional method for administering a drug is a single dose at high concentration, which will often result in systemic toxicity since it is above the therapeutic index. Repeated administration is troublesome and will not be continuously effective because the drug levels fluctuate in the blood plasma. In the case of chemotherapy, which distributes drugs to the whole body, the drug levels can fluctuate in such a way that it leads to unintended and unacceptable side effects. Thermogelling drug delivery systems improve patient compliance because multiple injections and frequent chemotherapy are not required.
- **High water content in the gel matrix improves biocompatibility.** An improved biocompatibility means that targeted delivery is more possible, but at the same time it is harder to control the release because of the nature of bulk erosion. Gels, with high content of water, can be used as a pH buffer and lubrication for drug delivery in sensitive body parts.¹⁸
- **Easy manufacturing in aqueous solvents.** This eliminates the need for organic solvents or radiation, which are common methods of crosslinking to form a gel. This means less denaturation of sensitive therapeutic agents, such as proteins or polypeptides.

4.5 Delivery of Insulin and Protein Drugs in the Treatment of Diabetes

Delivery of proteins or peptides remains a problem due to their short residence half-life and fast renal clearance.²² This problem is complicated by the burst release of small or medium-sized hydrophilic peptide drugs due to the lack of stability of thermogelling systems in the body, especially those which are biodegradable. However, many clinical therapeutic methods require a sustainable delivery of drugs. How can a biodegradable thermogelling polymer deliver these drugs at a constant rate yet remain stable at the target site? Using the treatment of diabetes as a prominent example, various strategies have been developed to deliver drugs and insulin at a sustained, constant dosage. Insulin is a polypeptide hormone which is naturally secreted by the body to regulate glucose metabolism. The structure is shown below. Since it is a polypeptide with -CONH- amide bonds, the insulin molecule is hydrophilic (Figure 4.3).

There are two types of diabetes. Type 1 diabetes is when the body cannot produce its own insulin. Type 1 diabetes treatment requires frequent supply of insulin, usually more than once daily, from external sources. Type 2 diabetes is when the insulin production is insufficient to lower blood glucose levels in the body due to insulin resistance, whereby cells fail to respond to normal levels of insulin. Type 2 diabetes is treated by various drugs to assist the absorption of insulin, and sometimes insulin injections are also required. Oral delivery of insulin, although most appealing to patients, suffers from various chemical, biochemical and physical barriers, such as acid-induced hydrolysis in the stomach, enzymatic degradation throughout the gastrointestinal tract, extensive first pass effects in the liver, bacterial fermentation in the colon and lack of permeation through the epithelial cells. A typical administration routine involves once, or more than once per day. Research on injection-based systems has concentrated on achieving a sustained release profile by providing additional protection to the drug

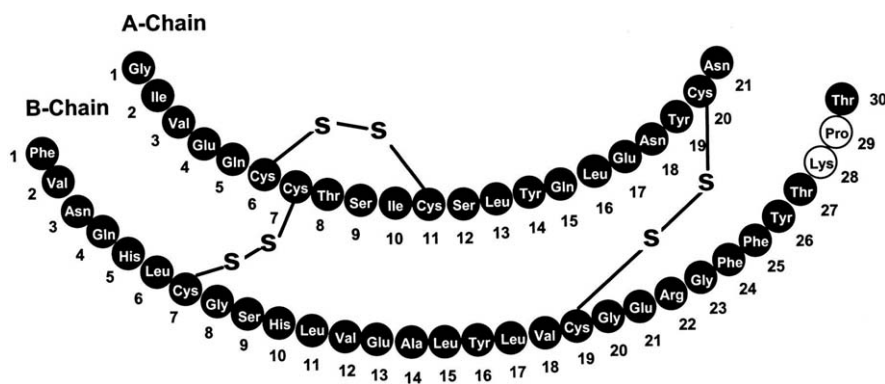


Figure 4.3 Chemical structure of insulin, with abbreviated amino acid components.

within the gel. Oak *et al.* mixed a PLA-PEG-PLA thermogel with chitosan–zinc–insulin complex for continuous *in vivo* insulin delivery at basal levels after a single subcutaneous injection. Chitosan–zinc–insulin effectively reduces burst release and protects the insulin from the acidic degradation products of the copolymer. The insulin release lasted for around 3 months in a streptozotocin-induced diabetic mouse without any immune response or change in structural integrity of the insulin.²³

Chen *et al.* developed a sustainable Liraglutide (drug for Type-2 diabetes) release system consisting of poly(ϵ -caprolactone-*co*-glycolic acid)-poly(ethylene glycol)-poly(ϵ -caprolactone-*co*-glycolic acid) (PCGA-PEG-PCGA) thermogels. They reported a sustained release for 1 week in mice, attributed to the amphiphilicity of Lira and the high chain mobility of the PCGA part at body temperature.²⁴ The delayed diffusion of drug was due to hydrophobic interactions between C16 fatty side chain of Lira and the hydrophobic cores of copolymer micelles. It was apparent from their experiment that an effective way of achieving sustainable delivery is by forming appropriate attractive interactions between the drug and the thermogel matrix.

One strategy for a more sustained release is by using synergistic excipients, such as the combination of zinc acetate, PEG and sucrose employed by Li *et al.* They managed to sustain the release of antidiabetic drug exenatide (a glucoregulatory drug) for 1 week from a PLGA-PEG-PLGA biodegradable thermogel mixture without burst release. They reported a synergistic effect between the PEG and sucrose to increase the viscosity of the thermogel and concomitantly act as porogens, which facilitate the later stages of exenatide release, while the zinc acetate inhibits burst release by forming insoluble Zn–EXT complexes within the thermogel matrix.²⁵ Additional *in-vivo* pharmacokinetic and pharmacodynamics studies confirmed that the sustained release of EXT from the gel matrix after a single subcutaneous injection improved the glucose tolerance in type-II diabetic mice for 1 week, with results comparable to the *in-vitro* drug release studies.

Ghasemi Tahrir *et al.* have reported the sustained release of human insulin over a period of 5 days by injecting a thermogelling chitosan/ β -glycerol phosphate solution subcutaneously into diabetic mice.²⁶ β -Glycerol phosphate is a salt that enables the formation of physical crosslinks between the chitosan chains *via* hydrogen bonding. The insulin incorporated into the chitosan/ β -Gp gel retained its structural integrity. Insulin is partially adsorbed onto the surface of the gel or suspended in the gel matrix during gelation and then diffuses rapidly when the gel encounters the body fluid.

4.6 Adaptation of Thermogels for Biomedical Applications

As an alternative to adding complexes or excipients, a completely new thermogel system with unique rheological properties can be made by modifying the chemical structure of the gel itself, leading to a change in the

degradability. However, many optimizations need to be investigated because the biocompatibility and the effectiveness of the resulting gel will be affected as well.

4.6.1 Selenium-containing Thermogels²⁷

Selenium was added to the hydrophobic end of PEG-PLGA to produce a triblock polymer Bi(PEG-PLGA)-Se, thereby eliminating end group effects common in ABA-type copolymers. The selenium atom in the selenium-containing thermogel can coordinate to the platinum of anti-cancer drug cisplatin, resulting in increased drug loading capacity. This additional interaction also contributes to the drug release because it introduces another release mechanism involving competitive ligands. Furthermore, by simply lengthening the coordination time, the initial burst release can be reduced.

4.6.2 Matrix Metalloproteinase-sensitive Thermogelling Polymers²⁸

Garripelli *et al.* studied Pluronic-based thermogels and incorporated an MMP2-sensitive peptide sequence such as GPLGIAGQ *via* oligomerization, to produce a low-MW biodegradable multiblock copolymer from a non-biodegradable Pluronic variant. This copolymer exhibited thermogelling characteristics at around body temperature. When MMP (overexpressed in most cancer cells) encounters and binds to MMP2-sensitive peptide segment, enzymatic degradation of the thermogel occurs. This provides an additional control mechanism for the rate of degradation. Subsequently, Garripelli *et al.* used the thermogel to deliver hydrophobic Paclitaxel *in vitro*, which accelerated release in MMP-containing media compared to MMP-free media.

4.7 Towards Understanding *In-vivo* Effectiveness of Polymeric Thermogel Drug Delivery

Despite potential advantages, the main reason biodegradable thermogelling polymeric drug delivery systems are yet to be commercialized is because the *in-vivo* or clinical trial performance is less than that of conventional drug delivery methods. This can be attributed to the difference between human and animal physiological models, unexpected localized side effects due to the polymeric component or additives, complex intercellular effects due to the particular drug concentration or release profile, and even differences between human patients. The design of these drug delivery systems is greatly complicated by these factors. Therefore, much research has been directed towards a deeper understanding of *in-vivo* performance.

4.7.1 Toxicological Aspects of the Use of Dextran Microspheres and Thermogelling Ethyl(hydroxyethyl) Cellulose as Nasal Drug-delivery Systems

Morath *et al.* investigated the effectiveness of insulin released by injecting ethyl(hydroxyethyl) cellulose (EHEC) through the mucosa in the nose. Interestingly, goblet cell hyperplasia and mild changes in the ciliary beat frequency were observed after repeated administrations of EHEC, which is attributed to the localized hypoosmotic conditions caused by the action of EHEC in the anterior part of the nasal cavity.²⁹ Following the administration of a particular thermogel, many parallel investigations need to be performed on different target sites to ensure the complete clinical safety of the thermogels.

4.7.2 *In-vivo* Pharmacological Evaluations of an Antioxidant-loaded Biodegradable Thermogel³⁰

Chow *et al.* functionalized a thermogel gelatin-*g*-poly(*N*-isopropylacrylamide) (GN) with antioxidant gallic acid (GA) using ascorbic acid and a free radical method. They then used this system to deliver the potent drug, pilocarpine, for the treatment of glaucoma (a retinal disease). The antioxidant in this system has a dual function. First, it helps to alleviate hypertension-induced oxidative stress in the eye, therefore reducing the likelihood of contracting glaucoma, where intraocular pressure (IOP) elevation is a significant risk factor. Secondly, the antioxidant molecules control the hydrophilicity of the thermogel, which in turn affects the rate of drug release due to different hydrolytic degradation rates in the body. Therefore, an additional method of controlling the drug release in this case is by changing the grafting temperature, which in turn affects the grafting amount of the antioxidant. These authors also carried out *in-vivo* studies in glaucomatous (experimentally induced) rabbits, investigating the biocompatibility and the drug release efficacy of the thermogel samples over time, and examined the corneal topography and electroretinogram measurements following the injection of the thermogel. Their results demonstrated that the incorporation of antioxidant molecules into the polymeric drug carrier could achieve antioxidative retinal cytoprotection in an animal model, retarding glaucoma development.

4.8 Conclusion

Biodegradable polymeric thermogels have the potential to replace conventional therapeutic methods as a drug delivery platform to treat diseases such as diabetes, glaucoma and cancer that require the sustained release of a therapeutic drug at a constant concentration. However, the vast array of possible polymers that can be customized and combined, together with our lack of understanding of the *in-vivo* performance of each means that

thermogel drug delivery systems are still a long way from commercialization. Nevertheless, progress has been made in animal models and the quest for a stable, biodegradable, non-toxic, slow release injectable or implantable sol-gel incrementally progresses.

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CHAPTER 5

Injectable Thermogelling Polymers for Bone and Cartilage Tissue Engineering

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5.1 Introduction

Tissue engineering is an interdisciplinary field with the goal of restoring functional tissue in the human body. This can potentially be achieved by combining a scaffold, cells, growth factors, proteins, and other biomolecules in such a way to achieve the target tissue. The choice of scaffold is crucial because it has an influence on cell proliferation, stem cell differentiation, and gene expression among other considerations. Ideally, the scaffold should mimic the 3D microenvironment of the embedded cell type, including mechanical properties, 3D structure and scaffold chemistry.

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Hydrogels are being intensively studied as scaffolds for tissue engineering. Hydrogels are cross-linked, insoluble, porous polymeric networks, which show a high water content, analogous to biological tissue. Injectable hydrogels are a subclass of hydrogels that gel under mild conditions at physiological pH and temperature. This enables physicians to carry out the gelling process *in situ* upon injection of the gel precursor into the patient's body. The fact that gelling occurs under mild conditions enables the mixing of cells and active biomolecules with the gel precursor solution before injection. The *in situ* gelling hydrogel fills out irregularities at the injection site. This approach shortens the medical operation time and minimises damage to surrounding tissues, reducing the patients' post-operational pain.¹

The gelling process, during which a polymer in solution becomes a gel, is referred to as the sol-to-gel transition.² Different mechanisms allow for an *in situ* sol-to-gel transition: photopolymerisation, chemical cross-linking, ionic cross-linking, and change in temperature, pH, or ionic strength.

Thermogels are hydrogels exhibiting the phase transition upon heating beyond a certain critical temperature, the lower critical solution temperature (LCST), or upon cooling below a certain temperature, the upper critical solution temperature (UCST).² This short review focuses on polymeric thermosensitive hydrogels exhibiting a LCST around body temperature. In this particular case, water is associated with the polymer chains below the LCST. The polymer is in solution. Usually, the hydrophobicity of the polymer chains increases during the heating process. The associated water is released and the polymer becomes insoluble. This leads to the formation of physical links forming a 3D polymeric network. We limit this review to applications in bone and cartilage tissue engineering, which have been the object of most recent research efforts. The following keywords in the *Scopus* database yielded 6837 papers, with the first published in 1973: [**gel**],[†] [*tissue*], [*engineering*]. We narrowed the search by including the keyword [*injectable*] and obtained 1061 results. Further narrowing the query to [*injectable*], [*thermo**],[‡] [**gel**], [*tissue*], [*engineering*] yielded 195 results (Figure 5.1)

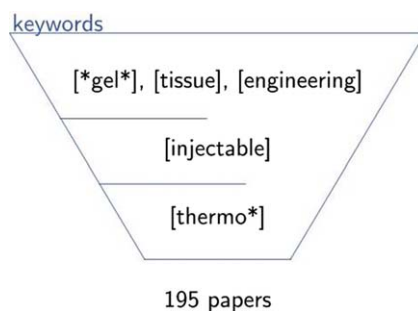


Figure 5.1 Literature research query.

[†]To include hydrogel, *etc.*

[‡]To include thermogels, thermoresponsive, thermosensitive, *etc.*

and 183 entries were found when excluding the keyword [**card**][§] from the search to avoid papers about applications for cardiac tissue engineering. Approximately 85% of these papers were published after 2007, and 50% were published in the last 5 years. These results highlight the interest in hydrogels for tissue engineering applications, and specifically injectable hydrogels.

5.2 Scaffold Requirements for Bone and Cartilage Tissue Engineering

Scaffolds have to meet particular requirements to be successfully employed in tissue engineering applications, as Kretlow *et al.*³ well described in their paper. Some of these are general requirements, while others are specific for bone or cartilage regeneration. The first and most important requirement is that the scaffold material be biocompatible. Not only the scaffold, but also leachable compounds and products of scaffold degradation must be biocompatible, as every cytotoxic effect is detrimental to the final application.

The scaffold should easily suspend cells in the polymeric solution and these should be retained during the phase transition and after gelation. The gelling process itself should occur under mild conditions at physiological pH and temperature, since it is carried out *in vivo* and the hydrogel is loaded with cells and active biomolecules. Harsh conditions might irreversibly damage the cells and biomolecules. The solid gel must have a certain degree of porosity and the pores should be interconnected. This provides space for the cells to grow and allows for the transport of nutrients to the cell and removal of waste products.

Bioactivity is another important parameter, to enhance cell proliferation, direct stem cell differentiation and promote overall tissue growth. Strategies to achieve scaffold bioactivity include the addition of bioactive particles (hydroxyapatite, bioactive glass, acellular bone matrix, *etc.*) or natural fibres (collagen, chitin, *etc.*) which mimic the extracellular microenvironment. The scaffold itself can be functionalised with proteins and signalling biomolecules, and growth factors can be mixed in to the polymeric solution. The scaffold and additives for bone tissue engineering should direct osteogenesis, during which, stem cells differentiate into osteoblasts, which then begin the process of new bone formation.⁴ In cartilage tissue engineering, the scaffold and biomolecules should promote chondrogenesis of the stem cells, *i.e.* the process by which cartilage is formed. Mesenchymal stem cells (MSCs) differentiate into chondrocytes, which then start to secrete the cartilage-specific extracellular matrix (ECM). Ideally, the scaffold will mimic the structure of natural cartilage.

Natural cartilage can be subdivided into several zones which exhibit different mechanical properties, chondrocytes morphology and chondrocytes orientation.⁵ Near the articular surface (Figure 5.2), in the superficial zone of the cartilage tissue, the chondrocytes are of ellipsoidal shape, parallel to the

[§]Cardiac, myocardium, *etc.*

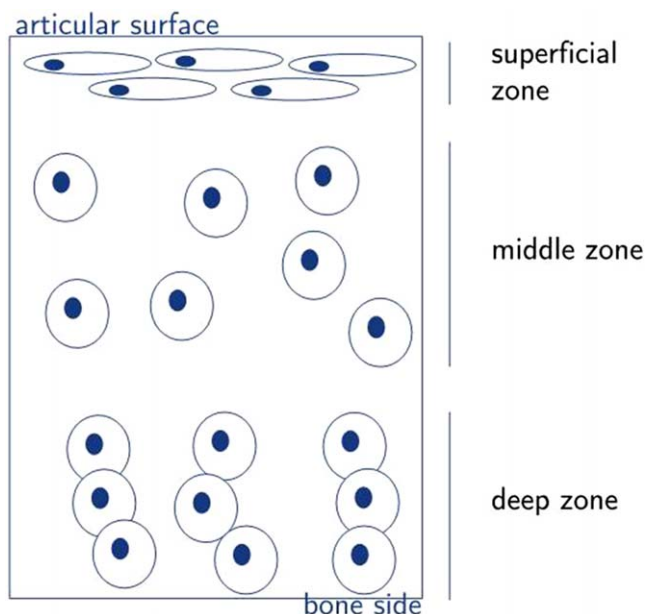


Figure 5.2 Schematic representation of natural cartilage and its different zones. Adapted from ref. 5 with permission from John Wiley and Sons, © 2016 Wiley Periodicals, Inc.

surface. In the middle zone, the cells are of spherical shape and randomly distributed in the bulk. This division correlates to cartilage functions and is vital for the performance of natural or engineered cartilage.

The mechanical properties of the scaffold are also important. The scaffold should sustain the tissue for initial growth and should match the mechanical properties of the natural tissue ECM. Natural cartilage is formed by a solid matrix soaked with a liquid. The mechanical properties depend on the cartilage water content. Natural cartilage has a Young's modulus between 450 and 800 kPa.⁶ On the other hand, natural demineralised bone, which represents the bone ECM prior to mineralisation, has a Young's modulus of around 300 MPa (femur 275 ± 94 MPa, tibia 351 ± 102 MPa.⁷ Moreover, the scaffold should be biodegradable with a degradation rate that ideally matches the rate of new tissue formation.

5.3 Chemistry and Properties of Selected Injectable Thermogelling Scaffolds

5.3.1 Totally Non-degradable Polymers

5.3.1.1 Poly N-Isopropylacrylamide

Some synthetic polymers and copolymers show LCSTs around physiological temperature and have been investigated as thermogels for tissue

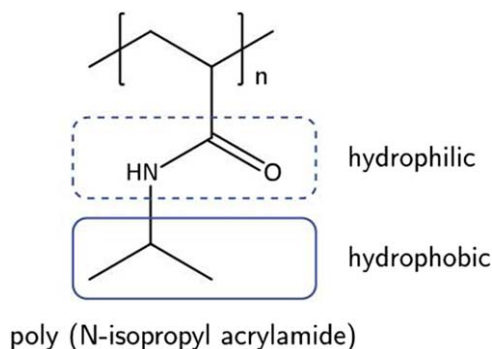


Figure 5.3 Simplified chemical structure of poly *N*-isopropyl acrylamide (pNiPAAm).

engineering. Poly *N*-Isopropylacrylamide (pNiPAAm) and its copolymers are good examples. This polymer exhibits a hydrophilic amide moiety and a hydrophobic isopropyl moiety (Figure 5.3). At low temperatures (below the LCST) water molecules are associated with the hydrophilic groups. pNiPAAm is therefore soluble due to extensive hydrogen bonding.² At higher temperatures, however, molecular motion exposes the hydrophobic groups, and hydrophobic interactions predominate causing the polymer (or copolymer) to precipitate in water. The sol-to-gel transition temperature can be controlled by changing the hydrophobicity of the comonomer. A more hydrophilic comonomer results in a higher sol-to-gel transition temperature due to extensive hydrogen bonding. Conversely, more hydrophobic comonomers lead to a lower LCST.

pNiPAAm and its copolymers are not biodegradable, which is a limitation for tissue engineering applications. Ideally, the scaffold should degrade at the same rate as the tissue grows. Thus, recent advances do not focus on pure pNiPAAm hydrogels but rather on composites or hybrids (e.g. graft-copolymers). Mellati *et al.* recently reported a study on the cytotoxicity of pNiPAAm⁸ and the use of pNiPAAm-chitosan composites⁹ and chitosan-graft-pNiPAAm copolymers⁵ for cartilage tissue engineering. Mellati *et al.*⁸ investigated the cytotoxicity of pNiPAAm of different molecular weights (MW) (monomer, P35, P100, P200, P400), where the number indicates the target degree of polymerisation. The cytotoxicity was assessed by the MTT assay. Cell viability was determined at 6, 24, 48, 72 and 96 h after incubation. The cytotoxicity tests with different cell lines yielded similar results for the same polymer used. The pNiPAAm monomer and the low MW polymer (P35) toxicity was concentration dependent, with a lower cell viability at higher concentrations. P35 and P400 were found to be cytotoxic only above a critical concentration, whereas P100 and P200 displayed high cell viability at all tested concentrations.

The authors provided a preliminary explanation for this behaviour. The small P35 polymer can freely flow at physiological temperature, since it possesses an LCST of ≈ 53 °C. It is therefore able to penetrate the cell

membrane and thus disrupt cell activity. The P100 and P200 already form large aggregates at 37 °C. They cannot penetrate the cells. P400 is thought to form a thicker, denser layer after the sol-to-gel transition. This layer might prevent nutrients and oxygen from reaching the cells underneath, thereby exerting a cytotoxic effect. Mellati *et al.*⁸ focused on the short-term cytotoxicity of pNiPAAm, but equally relevant to medical applications is long-term cytotoxicity of pNiPAAm, because a non-biodegradable polymeric implant resides in the body indefinitely.

In a later paper, Mellati *et al.*⁹ report a chitosan-pNiPAAm composite for cartilage tissue engineering. Chitosan is commonly used because of its chemical similarity to glucosaminoglycans (GAGs) and hyaluronic acid, two of the main components of natural cartilage. The authors infiltrated MSCs loaded with pNiPAAm into a porous chitosan scaffold to provide a 3D porous structure suitable for cell growth, proliferation and differentiation. The presence of hydrophilic amide moieties of pNiPAAm in the hybrid composite increased the water content of the whole scaffold. This improved the system with regard to nutrient delivery, waste removal, cell migration and ECM secretion. SEM micrographs, LIVE/DEAD assays and microscopy images showed that the thermogelling hydrogel well supported the 3D distributed cells. The scaffolds enabled culturing of cells in a 3D microenvironment resembling natural cartilage. However, the overall cell viability decreased after 7 days of culturing, although it was 108% higher in the composite compared with the pure chitosan scaffold. The authors hypothesised that the positive surface charge of chitosan might have a certain cytotoxic influence, similar to other positively charged polymers.

The authors studied the chondrogenic differentiation of the embedded MSCs. The biochemical assays showed a substantial DNA weight increase, and a 2.5-fold increase of GAGs and collagen secretion between 14 and 28 days. This indicated the suitability of a composite chitosan-pNiPAAm system for cartilage tissue engineering.

However, the system as a whole is not injectable since it requires a pre-formed chitosan scaffold. Moreover, the pNiPAAm gel infiltrated into the scaffold is injectable, but not biodegradable. Biodegradability studies of the composite *in vitro* and *in vivo* need to be performed to validate this approach. *In vivo* studies are also required to assess the host tissue response to this type of implant and to confirm the chondrogenic differentiation, and GAGs and collagen secretion in an animal model. In a later paper, Mellati *et al.*⁵ developed a chitosan-graft-pNiPAAm thermoresponsive hydrogel for cartilage tissue engineering. The main purpose of this new development was to tailor the cell orientation and morphology to mimic the different properties of natural cartilage (Figure 5.2). Lithographic techniques are suited to create micropatterned templates to direct cellular alignment. The authors first synthesised a chitosan-graft-pNiPAAm copolymer and investigated this system with regard to biocompatibility and chondrogenic differentiation. They tuned the polymer concentration to 2.5 wt% to achieve

the best mechanical properties[¶] without compromising the porosity and pore interconnectivity of the scaffold,^{||} which are needed for nutrient and waste transport. The mesenchymal stem cells were mixed into the polymeric solution, which was then gelled at physiological temperature. The cell number increased with culture time. The biochemical assays indicated a sixfold increase of GAGs between days 7 and 28, whereas the amount of collagen increased sevenfold over the same period. This observation suggests a successful chondrogenesis of the seeded MSCs in the 3D micro-environment provided by the investigated hydrogel.

Mellati *et al.* then fabricated micropatterned PEG-DMA micromolds *via* soft lithography. The authors investigated grooves of different widths (50, 100 and 150 μm). The cell-laden chitosan-graft-pNiPAAm solution was casted in the micromould and gelled at physiological temperature. The cells proliferated within the grooves and began to form a network at day 5. Fluorescence microscopy images with different dyes showed predominantly aligned elongated cell nuclei within the grooves.

These findings confirmed the authors' approach to mimic cell morphology and orientation found in natural cartilage. Further investigation is required to develop a functional piece of cartilage, which exhibits a morphology and orientation gradient as found in natural cartilage. Crucial to *in vivo* applications is to assess the biodegradability of the chitosan-graft-pNiPAAm copolymer scaffold as well as the immunological and cytotoxic compatibility of the polymer itself and its degradation products.

5.3.2 Enzymatically Degradable Polymers

5.3.2.1 Chitosan

Chitosan is a linear polysaccharide characterised by randomly distributed D-glucosamine and N-acetylglucosamine units (Figure 5.4). It is obtained through the partial deacetylation of chitin, which is found in the exoskeleton of crustacea. Its chemical structure resembles the GAG network, which is part of the cartilage ECM. It thus has been investigated as a component of scaffolds for cartilage tissue engineering.¹⁰

Chitosan is insoluble in water at physiological pH, but becomes soluble below a pH of 6.5, when $-\text{NH}_2$ groups are protonated.¹¹ Chitosan solutions gel in the presence of polyol salts at higher pH values. β -Glycerophosphate (β -GP) is known to induce a sol-to-gel transition in chitosan solutions at physiological pH and temperature.¹² A further advantage of chitosan for tissue engineering is its intrinsic antibacterial activity and low immunogenicity.^{11,12}

The mechanism of gelation was first proposed by Chenite and coworkers in the early 2000s.^{13,14} Adding β -GP salt to the acidic chitosan solution

[¶]Better mechanical properties with higher concentration.

^{||}Higher porosity and pore interconnectivity at lower concentrations.

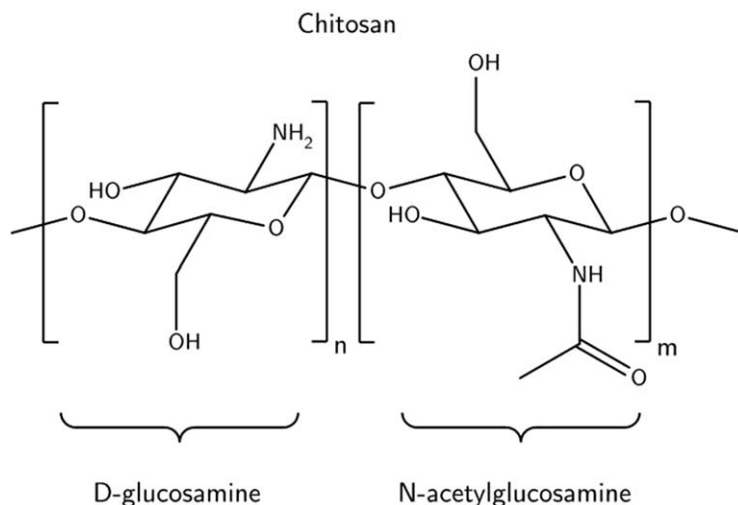


Figure 5.4 Simplified chemical structure of chitosan.

increases the pH to a physiological value (6.8–7.2), while maintaining the solubility of chitosan in water. The thermogelling behaviour of chitosan/ β -GP water solutions originates from increasing hydrophobic interactions between chitosan macromolecules at higher temperatures.¹³ At low temperatures, chitosan aggregation is prevented by the strong interaction with water molecules. Heating increases the hydrophobic interactions between chitosan chains. This is mediated by the presence of β -GP, which masks the positive charge of chitosan amine groups, due to the electrostatic interaction with its own phosphate moieties.¹⁴ The electrostatic repulsion between positively charged chitosan chains is effectively reduced.¹⁵ Noteworthy is that β -GP does not take part in the physical cross-linking of the chitosan hydrogel, as it can be easily washed out.¹⁴ Wang *et al.*¹² developed a chitosan and collagen type I composite thermogelling scaffold for bone tissue engineering. β -GP was chosen as the gelling agent, as it induces a sol-to-gel transition in chitosan and the authors hypothesised it would induce reconstruction of collagen fibrils by neutralising the acidic collagen type I solution. They successfully built composite scaffolds by increasing the solution temperature to 37 °C. Wang and colleagues reported Young's moduli of ≈ 18 kPa for chitin/collagen ratios of 65/35 and 25/75. Pore sizes in the scaffold ranged from 1 μm to 4 μm . The authors incorporated human bone marrow stem cells (hBMSCs) to assess cell viability, osteogenic differentiation, cytotoxic effects and further biochemical assays. They associated the presence of collagen in the scaffold with increased cell spreading and proliferation as well as with improved scaffold stiffness. In turn, chitosan was associated with increased osteogenic differentiation. The authors also reported a cytotoxic effect of β -GP, as it was found to inhibit cell metabolic activity as soon as 4 h after gelation. Washing the formed gel

effectively removed excess salt, but this precludes *in situ* gel formation. Moreira *et al.*¹⁶ also reported chitosan/collagen composite thermogelling scaffolds for bone tissue engineering using β -GP as the gelling agent. They investigated the incorporation of bioactive glass (BG) nanoparticles into the matrix as a method for promoting the formation of hydroxyapatite (HA), which is characteristic of mineralised bone tissue. They reported improved mechanical properties of chitosan-collagen composites compared to pure chitosan. The G' value increased from an average of 6.56 Pa to an average of 9.11 Pa (39% increase in stiffness). The incorporation of BG nanoparticles further increased the G' value to 12.77 Pa average (95% increase in stiffness compared to pure chitosan). The authors reported a sol-to-gel transition temperature of 37 ± 2 °C for the composite scaffold. In certain cases, this could lead to an incompletely formed gel at physiological temperature, causing the release of active biomolecules, cells or other loaded compounds. Moreira and colleagues performed MTT and LIVE/DEAD assays to assess cytocompatibility and the result confirmed the non-cytotoxicity of the composite scaffold biomaterial. A closer investigation of chitosan/collagen β -GP thermogelling systems might be needed as Wang *et al.*¹² reported inhibition of cell metabolic activity due to β -GP in a similar system. Long-term *in vivo* biodegradability studies of both systems would shed light onto the host tissue response to an implant from the proposed formulations. The investigation of inflammation caused by degradation byproducts is fundamental to further validate these strategies for bone tissue engineering.

5.3.3 Hydrolytically Degradable Polymers

5.3.3.1 PEG and PLGA derived Copolymers

Poly ethylene glycol (PEG)- and poly lactic-co-glycolic acid (PLGA)-derived copolymers have been extensively studied as biodegradable materials for biomedical applications. Jeong *et al.*¹⁷ reported as early as 1999 the thermoreversible gelation of PEG-PLGA-PEG triblock copolymers. Jeong and colleagues investigated the degradation behaviour of this material in a later paper.¹⁸ Jeong *et al.*¹⁷ proposed the following gelation mechanism: PEG-PLGA-PEG triblock copolymers (BAB-type) exhibit a sol-to-gel transition temperature which is dependent on the relative length of the blocks. The copolymer spontaneously forms micelles in water (Figure 5.5). The PLGA is located in the core, while the PEG represents the shell. As temperature increases, polymer-polymer attraction increase and micelles grow. The larger micelles contact each other and the PEG block interpenetrates neighbouring micelles. The polymer-polymer interaction prevents the dissolution of the newly formed aggregate. Above a certain micelle concentration an insoluble network is formed.

The gelation mechanism for PLGA-PEG-PLGA triblock copolymers (ABA-type) is similar, although it occurs at lower temperatures compared to

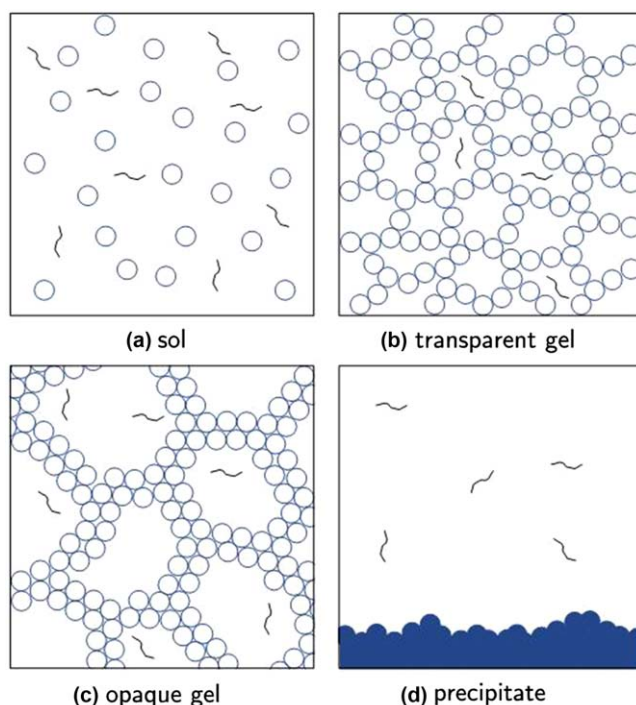


Figure 5.5 Schematic representation of the gelation steps of PLGA-PEG-PLGA. Temperature increases from steps (a) to (d). (a) is below the LCST, while (d) is above the UCST. Reprinted with permission from ref. 19. Copyright 2016 American Chemical Society.

BAB-types. The hydrophobic blocks can be located in neighbouring micelles, effectively bridging them.¹⁹ This makes the aggregation easier and shifts the onset of gelation to lower temperatures.

PLGA-PEG-PLGA triblock copolymers have been investigated for use in tissue engineering. Zhang *et al.*²⁰ mixed bone marrow mesenchymal stem cells (BMMSCs) into a PLGA-PEG-PLGA triblock copolymer solution with the aim of chondrogenesis and stem cell differentiation to initiate cartilage repair. They reported good adhesion of cells to the scaffold after gelation and observed proliferation of cells over a period of 21 days. The 3D porous network represented an ideal environment for cell growth. The scaffold showed good mechanical properties. A G' value of 1 kPa at 37 °C was measured after rinsing the gel with phosphate-buffered saline (PBS) to simulate the mechanical properties *in vivo*. The gel degraded within 42 days *in vivo*, while it showed different degradation rates *in vitro* depending on the medium it was exposed to. The gel fully degraded in proteinase-K and elastase after 15 and 50 days, respectively. In PBS, 75% of mass was still present after 70 days. The inflammatory response *in vivo* was assessed. The

authors reported an acute inflammatory response after 7 days (visual field filled with granulocytes), which turned into a chronic inflammation at 14 days (presence of macrophages and lymphocytes). After 42 days, as the gel biodegraded completely, the inflammation disappeared. Gohil *et al.*²¹ proposed a formulation for a composite injectable hydrogel for bone tissue regeneration. As mentioned above, scaffolds designed for bone tissue engineering should direct osteogenesis. Ideally, the scaffolds are osseointegrating, osteoconductive and osteoinductive, promoting osteogenesis and the biomineralisation of the tissue. Hydroxyapatite (HA) and tricalcium-phosphate (TCP) are ceramic biomaterials extensively studied as osteoconductive materials as well as materials to promote osseointegration.²² Advances in synthetic peptides have led to the development of biomimetic bone matrix components. The peptide P-15 replicates an amino acid sequence found in the $\alpha 1(I)$ chain of type I collagen.²³ P-15 was found to promote cell differentiation, enhance ECM secretion and aid the overall bone regeneration process. Gohil *et al.*²¹ incorporated nano hydroxyapatite (n-HA) crystals and P-15 peptide into a thermoresponsive PLGA-PEG-PLGA matrix. They further observed that the n-HA content had an influence on the gelling behaviour of the composite, causing loss of mechanical integrity above a concentration of 18.2% (wt/wt). Although the concept behind the paper is very interesting, further research is required to validate this approach to bone tissue engineering. Rheological studies should be performed over a temperature range (e.g. 4–50 °C) at different n-HA contents to characterise the sol–gel transition temperature of the matrix. An investigation of porosity is crucial to assess the potential use of the system, as interconnected pores are required for nutrient delivery and waste removal. Moreover, biodegradability studies *in vitro* and *in vivo* are needed to compare the rate of degradation of the matrix with the rate of new tissue formation. Further investigation is needed to assess cytotoxicity of the scaffold formulation loaded with, for example MSCs. Extensively used assays such as the LIVE/DEAD, the MTT and the CCK-8 assay will also give insight into cell proliferation, cell distribution and morphology.^{16,24} To assess osteogenesis, an investigation of the bone-specific cell products such as collagen type I content, calcium content and other biomolecules is required.

5.3.3.2 PEG- and PCL-derived Copolymers

PEG- and poly ϵ -caprolactone (PCL)-derived copolymers have also been the object of recent research efforts in the field of biodegradable thermogelling materials for tissue engineering and drug delivery.^{25,26} The development of these materials has been driven by the sticky nature of PEG-PGLA-based systems when dry. This makes the handling and weighing after lyophilisation problematic. A dry, flowing powder-like system would be much preferred.

Hwang *et al.*²⁵ first reported a PEG-PCL-PEG BAB-type triblock copolymer. The crystalline nature of PCL renders this system powder-like after

lyophilisation. The dry PEG-PCL-PEG is easily redissolved in hot water at 50 °C (10 s) followed by quenching at 0 °C (30 s). PEG-PCL-PEG shows a sol-to-gel and a gel-to-sol transition similar to those of PEG-PLGA-PEG. The lower transition happens due to micellar aggregation, analogously to the PEG-PLGA system. The authors found that the upper transition was governed by increased molecular motion of the PCL segment at higher temperatures. Hwang and colleagues studied the effect of segment length on the phase diagram of this class of gels. In short, increasing the PEG segment molecular weight moved the LCST to higher temperatures. If the PEG segment was too long compared with the PCL segment, the sol-to-gel transition happened out of the range of interest for biomedical applications. Likewise, if the PCL segment was too long, the triblock copolymer could not be dispersed in water. The length of the hydrophobic block determined at some point the (in)solubility of the compound.

Bae *et al.*²⁶ developed the ABA-type PEG-PCL system. In the case of PCL-PEG-PCL, the gel transition is driven by micellar aggregation as in the PEG-PGLA-based system. Increasing the PCL molecular weight shifts the sol-to-gel transition to lower temperatures and the gel-to-sol transition to higher temperatures. The gel window therefore increases. A higher PEG molecular weight shifts the whole phase diagram towards higher temperatures. Moreover, in this case, the system is powder-like after lyophilisation.

The authors compared both the PEG-PCL-based ABA and BAB systems. The results are based on triblock copolymers with a similar amount of PEG/PCL and a comparable PEG/PCL ratio (PCL-PEG-PCL: 980-1000-980, PEG-PCL-PEG: 550-2190-550; the numbers represent the segment molecular weight). The BAB type forms regular micelles with a hydrophobic core and a hydrophilic shell. The ABA type presents micelles with loops and bridges, as the polymeric building blocks have two hydrophobic segments at each end. The micelle bridging in the ABA type renders micelle formation easier, as is the case for the PEG-PLGA system. The sol-to-gel transition occurs at lower temperatures compared with PEG-PCL-PEG, and the gel-to-sol transition occurs at higher temperatures (Figure 5.6). Thus, the gel phase is observed in a wider temperature range.

The authors also observed an influence on the storage modulus (G') of the gel. The ABA type shows a higher storage modulus (10 000 Pa) compared with the BAB type (100 Pa). The improved mechanical properties and the broader gel temperature range render the ABA-type PCL-PEG-PCL better suited for an injectable system for tissue engineering. The copolymer was powder-like once lyophilised and could be easily redissolved in water above the melting temperature of PCL followed by quenching at 0 °C. Wang *et al.*²⁷ attempted to improve the ABA-type (PCL-PEG-PCL) system by eliminating the need for heating the system to ensure complete dissolution of the polymer. They hypothesised that reducing the PCL crystallinity would improve the dissolution process. The authors incorporated a pendant cyclic ether group into the PCL segment *via* the ring opening polymerisation of 1,4,8-trioxo[4.6]spiro-9-undecanone (TOSUO) and ϵ -caprolactone in the presence

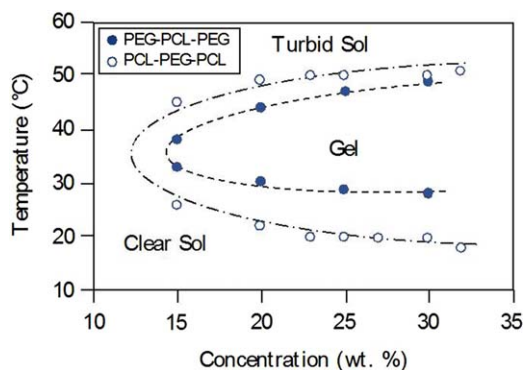


Figure 5.6 Effect of topology on the phase diagram of triblock copolymer aqueous solutions. Phase diagrams of PEG-PCL-PEG (550-2190-550) and PCL-PEG-PCL (980-1000-980) were compared. The precision of the measurements was within ± 1 °C.

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of PEG (Figure 5.7). The triblock copolymer effectively became PTOUSO-*b*-PCL-*b*-PEG-*b*-PCL-*b*-PTOUSO, where the two PTOUSOs and the two PCLs had the same length, respectively. The authors reported an easier dissolution in water after the incorporation of TOSUO into the PCL segment. Increasing the length of the TOSUO segment lowered the LCST as the overall molecular weight of the hydrophobic building block increased. This is the same effect reported by Jeong and coworkers.^{25,26} However, compared with the unmodified PCL-PEG-PCL system, the storage modulus G' reported by Wang *et al.*²⁷ was three orders of magnitude lower (45 Pa, compared to 10 000 Pa reported by Bae *et al.*²⁶). The cumbersome modification simplifies an aspect of the system, but reduces its mechanical properties.

5.4 Conclusions

In this short review paper we have focused on the developments of injectable thermogels for cartilage and bone tissue engineering. We have covered pNiPAAm-, Chitosan-, PEG/PLGA- and PEG/PCL-based hydrogels. Generally, effort has been directed towards the development of systems which meet the requirements of cartilage and bone tissue engineering applications. Often, the authors of the papers we have reviewed focused their research on novel materials or improvements of existing materials, performing short-term cytotoxic and biocompatibility studies. The success of novel materials for tissue engineering relies on regulatory compliance (*e.g.* FDA approval). The development of new materials should therefore include extensive *in vitro* and especially *in vivo* studies. These are necessary to assess the effects of the hydrogel on the host tissue and the effectiveness of these materials as injectable scaffolds. Parameters and properties to monitor in this regard

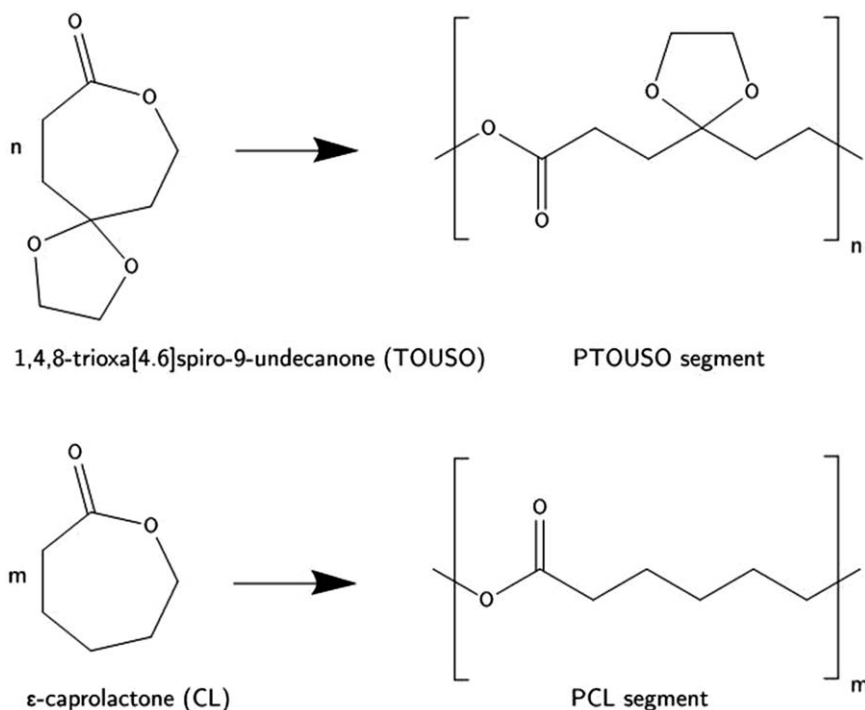


Figure 5.7 Polymerisation of 1,4,8-trioxaspiro[4.6]undecanone (TOSUO) and ε-caprolactone. Wang *et al.*²⁷ used both PTOUSO and PCL as segments in the hydrophobic blocks.

are inflammatory response, cytotoxicity, biodegradability, injectability, osteogenesis and chondrogenesis, degree of new tissue formation, and ECM secretion, among others. Too often cytotoxicity is assessed for a limited period of time. Ultimately, however, the whole lifetime of the implanted scaffold, from injection to complete degradation, must be investigated. These long-term studies are costly and time-consuming, but they are required to bridge the gap between a promising discovery and a commercial application.

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CHAPTER 6

Thermogels for Stem Cell Culture

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6.1 Introduction

The culturing of adult stem cells is one of the main foci of tissue engineering because of the capacity of these cells to differentiate into different kinds of tissue. The stem cell properties of self-renewal, homing to abnormal sites, suppressing of the immune response, and cell differentiation have therapeutic potential in regenerative medicine.^{1,2} The ultimate goal of this therapy is a procedure whereby stem cells are injected into a trauma site with localized cell proliferation and differentiation to heal the particular tissue or organ and thereby avoid major surgery. However, *in-vivo* stem cell proliferation is a relatively new area of science. Stem cell growth and differentiation are affected by many factors that are not fully understood.

This chapter will explore one important factor for *in-vivo* stem cell proliferation, which is the suitability of the matrix or scaffold for the cells to

grow and differentiate. The physicochemical characteristics of the scaffold have been found to be important in this respect. Properties such as chemical functional groups, stiffness, shape and size of patterned substrate and incorporated fillers have been shown to affect stem cell differentiation.^{3–5} Stem cells have traditionally been cultured in a conventional 2D environment (*e.g.* petri dish) which does not represent the 3D *in-vivo* environment.⁶ Development of bio-inspired 3D scaffolds that can direct stem cell differentiation is crucial to further study of stem cell therapies.

Biodegradable thermogels are potential injectable 3D scaffolds for regenerative medicine. Thermogelling polymers can be designed to transform from a sol to a gel at body temperature. Stem cells and additional growth factors can therefore be mixed with the aqueous solution of the thermogel and can be injected into the host. The cells are then suspended in the thermogel scaffold after gelling. The injected sol polymer is able to form in any irregular cavity shape in the targeted area without the need for large-scale surgical procedures. The biodegradable thermogel then degrades after the cells have grown to their respective adult cells and are able to sustain themselves. Thermogelling systems provide more benefit than typical cross-linking systems that require either radiation by UV light or chemical initiation, which may affect the proliferation of the stem cells.^{7–9}

Several research papers have tested different types of thermogels for their viability to not only provide a scaffold for proliferation, but also to induce the desired differentiation. Stem cells are required to differentiate into particular, functional adult cells (*e.g.* for bone tissue replacement, the stem cells have to undergo osteogenesis to form bone marrow cells rather than other types of differentiation).

6.2 Thermogel 3D Scaffolds for Proliferation and Chondrogenic Differentiation of Stem Cells

Min Hee Park *et al.* conducted a study on encapsulating tonsil-tissue-derived mesenchymal stem cells (TMSCs) in poly(ethylene glycol)-poly(L-alanine-co-L-phenyl alanine) (PEG-PAF) thermogel.¹⁰ The PEG-PAF thermogel is known to be easily degraded by certain enzymes and exhibits no change in acidity. This thermogel system has also been successfully employed for the sustained release of insulin and growth hormone.¹¹ In this experiment, TMSCs suspended in PEG-PAF were injected into the subcutaneous layer of mice. Adipogenic, osteogenic, and chondrogenic factors were added to determine the preferred differentiation route of TMSCs cultured in 3D PEG-PAF scaffold. The PEG-PAF thermogel was prepared from MW 650 PAF and MW 1000 PEG. The resulting mixture exhibited a gel modulus of 100–150 Pa at 37 °C. The gel was stiff enough to hold the stem cells inside the scaffold and able to retain enough durability to last for 3 weeks. The sol–gel transition was driven by micelle aggregation caused by partial increase of β -sheet content of PAF block and a partial dehydration of the PEG block.

The TMSCs were cultured using specific media supplements that induce adipogenesis (A), chondrogenesis (C), osteogenesis (O), and one control media without any supplements by using a basal growth medium (G). After 21 days, the cell densities in all media increased about two times, indicating a successful proliferation of TMSCs (Figure 6.1a). By staining the cells with calcein AM, differences in the cells' morphologies could be observed. After 21 days of incubation, small fiber-like protrusions appeared around the spherical shapes of the cells (Figure 6.1b). These fibrous branches were more prominent on cells cultivated with chondrogenic supplements (Figure 6.2). TMSCs usually prefer adipogenic rather than chondrogenic differentiation

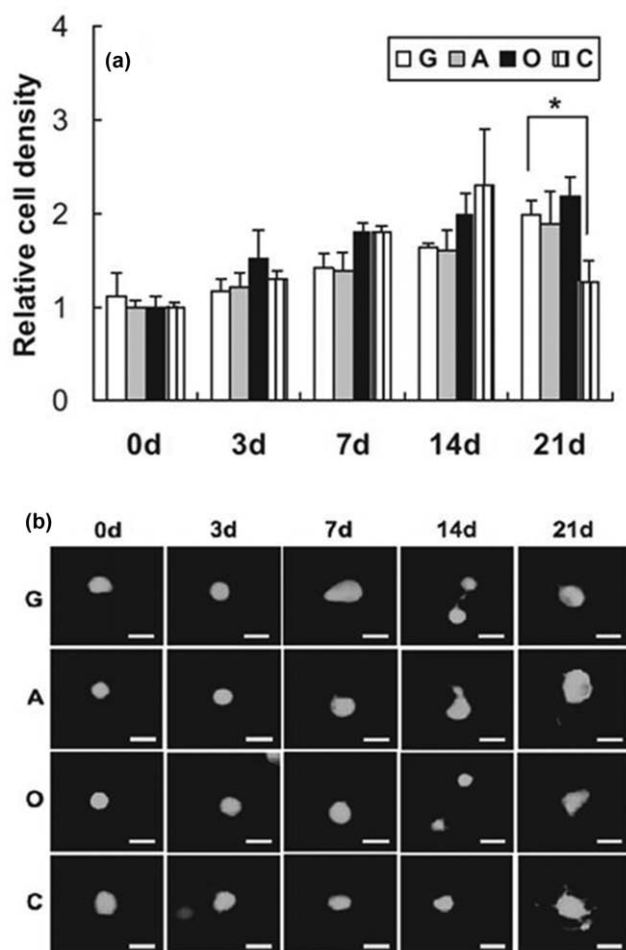


Figure 6.1 (a) Cell density, assayed using Live/Dead image analysis. (b) The observed morphologies of cells cultivated in different media.¹⁰ Reproduced from ref. 10 with permission from John Wiley and Sons, © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

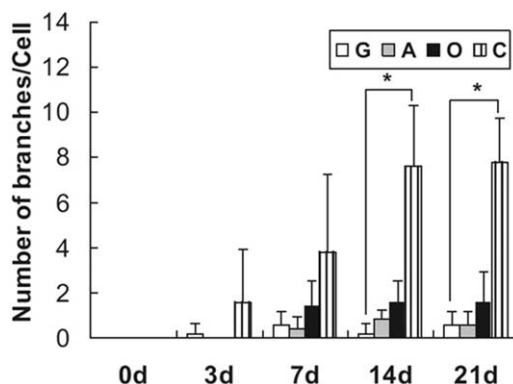


Figure 6.2 Number of branches per cell after certain days.¹⁰
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and maintaining its spherical shape without any branches.¹² These observed changes might indicate changes in the TMSC's preferential differentiation lineage.

To further investigate the differentiation route taken by these cells, the tissue-specific extracellular matrix (ECM) of the hydrogel used for the cultivation was analyzed by evaluating the peroxisome proliferator-activated receptor-gamma (PPAR γ), osteocalcin (OCN), and collagen type II (Col II) levels. These are indicators or biomarkers of mRNAs that are expressed during adipogenesis, osteogenesis, and chondrogenesis, respectively. The markers were stained with red immunofluorescence so that if any of those chemicals appeared they could be easily observed. After 21 days of 3D culture, Col II was the only one that showed a very obvious staining (Figure 6.3).

This test indicated that stem cell differentiation can be affected by the 3D scaffold used to cultivate the cells. The PEG-PAF thermogelling system proved to be good at inducing chondrogenic differentiation, as indicated by the high expression of Col II and sulfated glycosaminoglycans (sGAGs). However, the exact reason for this preferential differentiation is not known. Suggested reasons included the stiffness and density of the gel network, cell morphology, and also the functional groups that exist in the PEG-PAF, but further research is required for confirmation. PEG-PAF is one thermogel that can be used efficiently as a 3D *in-vivo* scaffold that induces chondrogenic differentiation.

Another study conducted by Hongjie Huang *et al.* used chitosan hydrogel as the 3D scaffold.¹³ Chitosan hydrogels have high biocompatibility, mix easily with cells and growth factors, and also retain the cells in the gel state.¹⁴ In this study, they used a hybrid scaffold made from chitosan thermogel mixed with demineralized bone matrix (DBM), which allows the scaffold to retain more cells homogeneously and gives better overall

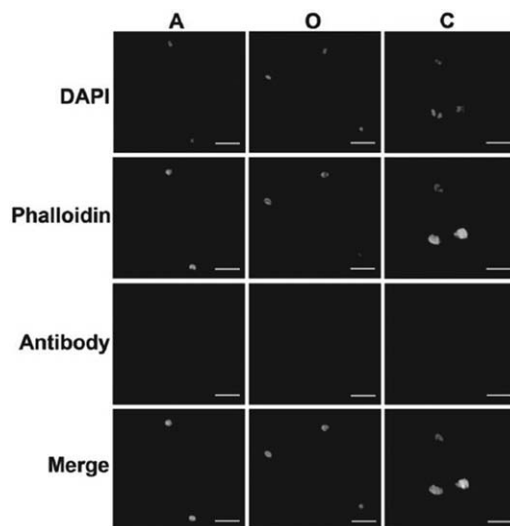


Figure 6.3 Immunofluorescence staining of PPAR γ (A), OCN (O), and Col II (C). DAPI and Phalloidin staining is used to stain the nucleus and action of a cell, respectively. The corresponding antibodies that bind to each protein are also used for staining. The scale bar is 20 μm .¹⁰ Reproduced from ref. 10 with permission from John Wiley and Sons, © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

structural strength, combining the properties of a gel and a solid ECM. The authors compared the proliferation and differentiation of stem cells cultivated in chitosan alone, DBM alone, and in a chitosan–DBM matrix.

The stem cells used were bone-derived mesenchymal stem cells (BMSCs) obtained from Sprague Dawley rats. After 72 hours of culture, the live/dead assay indicated that all three scaffolds were able to support live cells. However, the CS–DBM scaffold was better at sustaining a 3D environment compared with CS and DBM alone, which after 3 days of culture showed a 2D cell-rich field at the bottom of the petri dish due to gravity. Cytoskeleton immunostaining was used to analyze the cell proliferation. Cells in the CS and CS–DBM had more fibrous structures, which indicates high chondrogenicity. The sGAG content of each scaffold was used to measure the rate of chondrogenesis. After 3 weeks in culture, the CS–DBM hybrid culture was found to contain more sGAG deposits than the other two.

The biomimetic scaffold made using chitosan and DBM successfully mimicked the structure of native cartilage. The porous hydrogel allows liquid and nutrients to flow easily throughout the scaffold, while the solid ECM provides structural support within the collagen network. This experiment further supports the theory that the structure of a scaffold and culture environment has a significant effect on stem cell differentiation, in this case, chondrogenic differentiation. However, this experiment did not explore the feasibility of *in-vivo* use of the thermogel scaffold.

6.3 3D Thermogel Scaffold for Proliferation and Osteogenic Differentiation of Stem Cells

A scaffold to potentially enhance osteogenic differentiation was investigated by Hyo Jung Moon *et al.* using PEG-PAF thermogel with calcium phosphate incorporated into the solution.¹⁵ The calcium phosphate crystals were added to give hard surfaces that mimicked real natural bone structures on which the stem cells could grow. The mesocrystals of calcium phosphate have different shapes: rod-like (R), flower-like (F), and nano-particles (S) (Figure 6.4). This experiment tested the three different mesocrystals for their effectiveness in stem cell proliferation and osteogenic differentiation.

TMSCs were the choice of stem cell used in this experiment, and cell proliferation using the thermogel system was successful. After 14 days of cultivation, the cell densities increased about 1.5 times for all crystal shapes (Figure 6.5a). Fibroblast morphology after 21 days in cultivation (Figure 6.5b) suggested successful osteogenic differentiation.

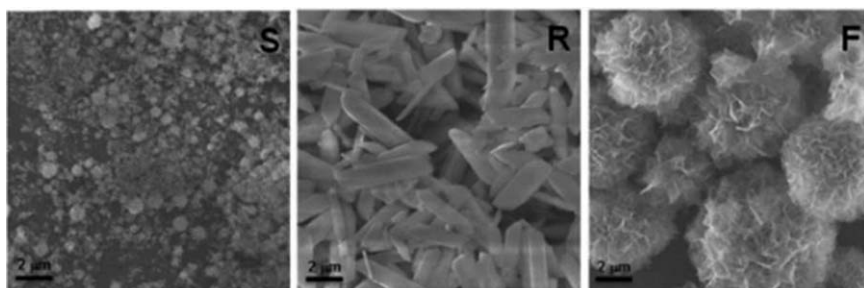


Figure 6.4 SEM image of calcium phosphate crystals.¹⁵
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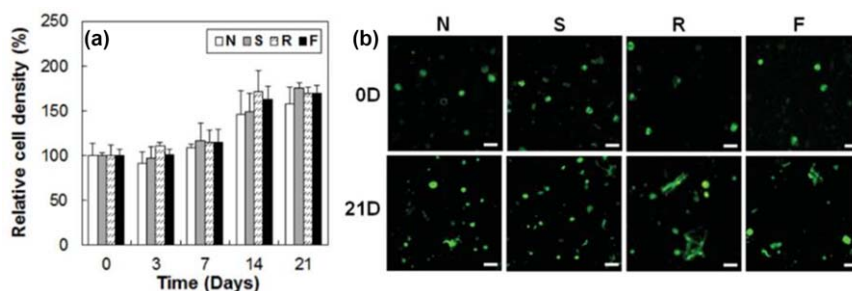


Figure 6.5 (a) Relative cell density determined by live/dead assay. (b) Immunofluorescence imaging of the TMSCs.¹⁵
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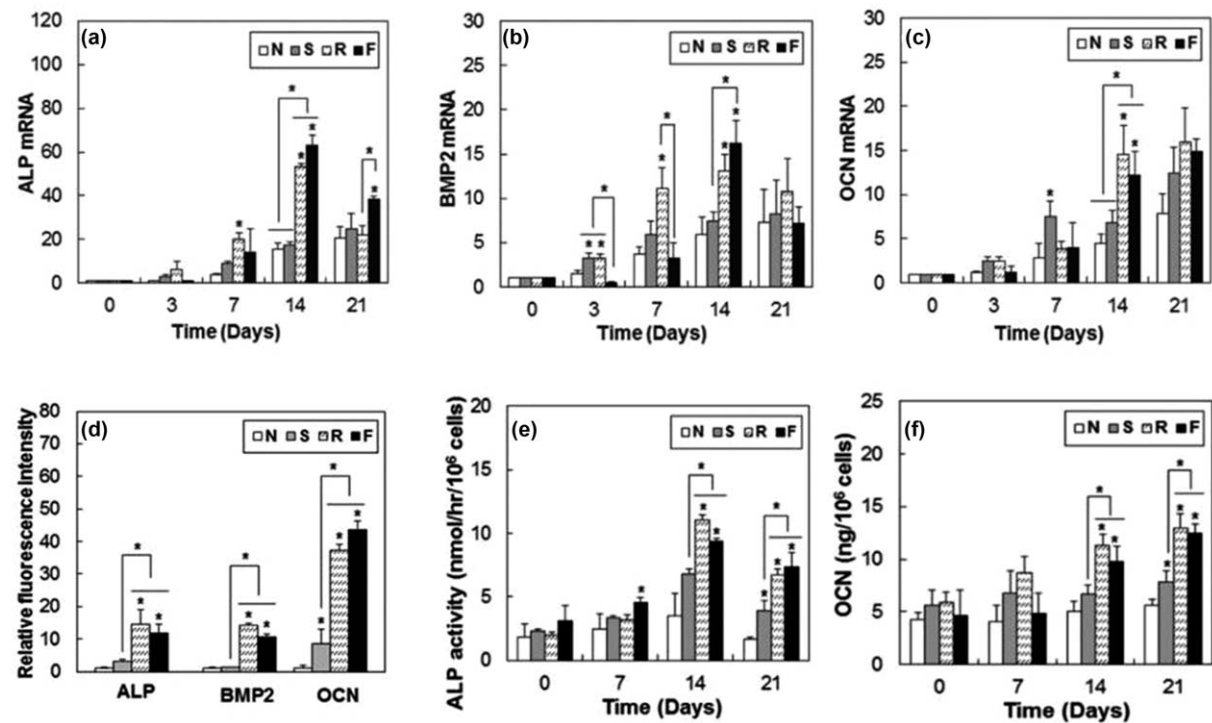


Figure 6.6 (a–c) The amount of mRNA genes expressed for its corresponding protein that indicates osteogenesis. (d–f) Amount of osteogenesis related protein detected using immunofluorescence imaging.¹⁵ Reproduced from ref. 15 with permission from John Wiley and Sons, © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Further analysis was performed by measuring the amount of osteogenesis-related mRNA expressed by the cells and also the resulting proteins translated from that mRNA. The osteogenic markers used were alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), and OCN. After 21 days of cultivation, all these biomarkers showed a significant increase (Figure 6.6a–f). ALP and BMP2 are enzymes that are more dominantly expressed in a developing skeleton and are usually made early in the development process. The ALP and BMP2 activity in the cells incubated in R and F was significantly higher than that in the N and S systems. OCN production of the R and F was also higher than for the N or S systems. The composite system of PEG-PAF with calcium phosphate mesocrystals (especially rod-like and flower-like crystals) was effective for enhancing osteogenic differentiation of TMSCs.

6.4 Thermogel 3D Scaffold for Proliferation and Adipogenic Differentiation of Stem Cells

Madhumita Patel *et al.* performed an experiment using graphene oxide (GO) and PEG-PA composite thermogel (GO/P) for adipogenic differentiation of TMSCs.¹⁶ Graphene and GO has been proven to enhance osteogenesis and adipogenesis of MSCs, respectively. This enhancement was attributed to the interaction between the material surface and proteins in the culture media.^{16,17} In this experiment, TMSCs and GO were suspended in PEG-PA aqueous solution. The mixture was then heated to 37 °C to form a 3D thermogel scaffold.

Cell proliferation was analyzed using the live/dead assay (Figure 6.7a and b). TMSCs were able to proliferate in all systems. However, TMSCs cultivated in GO/P displayed cell spreading that is usually associated with adipogenesis

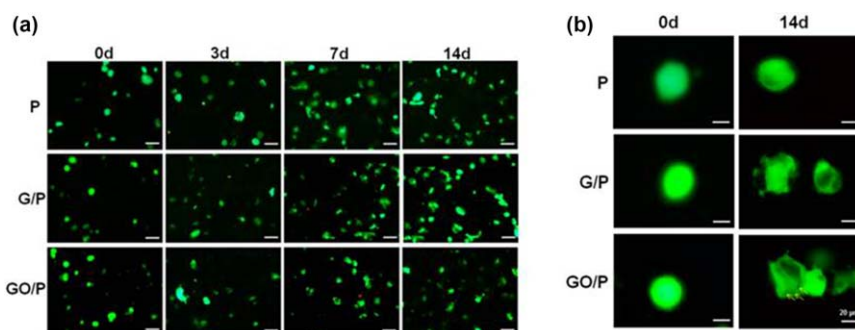


Figure 6.7 (a) Immunofluorescence LIVE/DEAD assay of TMSCs in a polymer only scaffold (P), polymer with graphene (G/P), and polymer with graphene oxide (GO/P). (b) Close up view of the TMSCs.¹⁶

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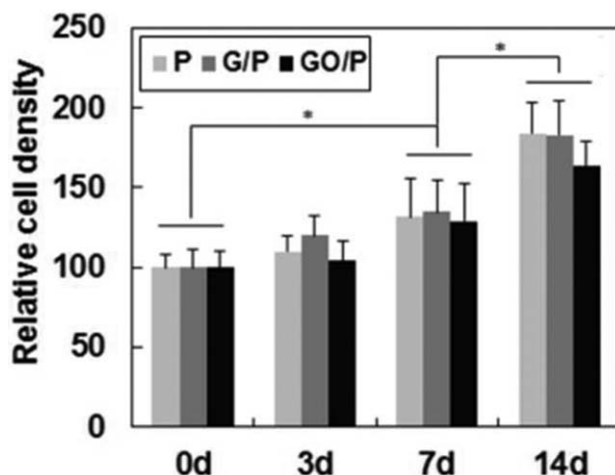


Figure 6.8 Relative cell density calculated using image analysis of the live/dead assay.¹⁶
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(Figure 6.7b, comp 41). The image was analyzed to measure the cell density and the results indicated a 1.5- to 1.8-fold increase in cell density after 14 days of culture (Figure 6.8). All three scaffolds were able to sustain cell growth and proliferation efficiently.

The adipogenic markers used in these experiments were peroxisome proliferator-activated receptor- γ (PPAR- γ), CCAAT-enhancer binding proteins- α (CEBP- α), lipoprotein lipase (LPL), adipocyte fatty acid binding protein 2 (AP2), elongation of very long chain fatty acids like-3 (ELOVL3), hormone-sensitive lipase (HSL), and uncoupling protein 1 (UCP1). After 14 days of cultivation, all biomarkers showed a significant increase and GO/P was at the highest concentration relative to the others (Figure 6.9). The GO/P composite system proved to be an efficient 3D scaffold for cultivating TMSCs and enhancing adipogenic differentiation.

6.5 Conclusion

Thermogelling composite systems show promise as 3D scaffolds for stem cell proliferation and differentiation. Thermogels alone can be used as a cultivating media. However, certain modifications are needed for stem cells to differentiate properly (*e.g.* graphene oxide for adipogenesis and calcium phosphate crystals for osteogenesis). The experiments described here have succeeded in cultivating and differentiating the stem cells to their respective adult cells. However, before the 3D injectable scaffold can be used *in-vivo*, further research is needed to test its viability for *in-vivo* injection, proliferation, and differentiation.

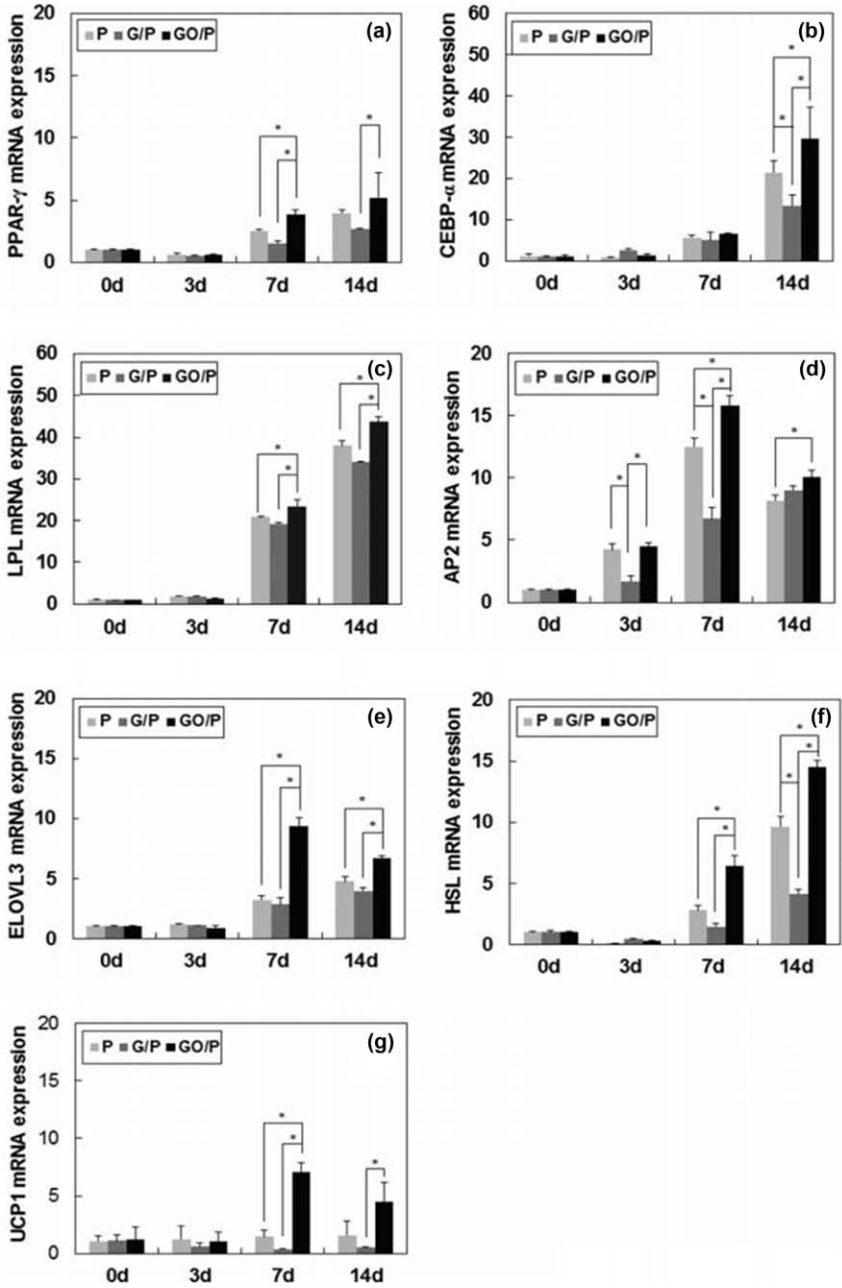


Figure 6.9 The concentration of mRNAs expressed that is associated with adipogenesis of TMSCs.¹⁶
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CHAPTER 7

*Degradation Behaviour of Biodegradable Thermogels*PEI LIN CHEE,^a DAVID JAMES YOUNG^b AND XIAN JUN LOH^{*a}^a Institute of Materials Research and Engineering, A*STAR (Agency for Science, Technology and Research), 2 Fusionopolis Way, Innovis, #08-03, Singapore 138634, Singapore; ^b Faculty of Science, Health, Education and Engineering, Maroochydore, Queensland 4558, Australia*Email: lohxj@imre.a-star.edu.sg**7.1 Introduction**

In 1986, Samejima and coworkers discovered that heating myosin achieved a strong gel with uniform porosity and fine filaments.¹ This discovery triggered a series of studies investigating the potential use of such systems. Egresi and fellow researchers used it to encapsulate microbial cells.² Kan, Doherty and Barron explored its application as a DNA sequencing matrix for large quantity electrophoresis analyses.³ Other researchers tested its application as a protein carrier.⁴ Physical hydrogels, such as those mentioned above, can undergo a sol-to-gel transition by a change in physical interactions between their component macromolecules, on change of temperature. This change in micelle arrangement in response to temperature determines the gelation efficacy. The basis of thermogelation lies in the miscibility of the starting solution. It can either have an upper critical solution temperature (UCST) or lower critical solution temperature (LCST). In the scenario of a thermogel that possesses a UCST, the components of the mixture are miscible above the identified temperature, whereas if the thermogel possesses an LCST, the components are soluble below this

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temperature. However, a UCST is less common for thermogels. Gels that possess the UCST are restricted in their practical applications as the components of the mixture are only miscible above the UCST and, for example, the high temperature can denature thermosensitive drugs.

The objective of this review is to highlight the importance of degradability for thermogel applications. Relevance of thermogels, techniques to tune their degradability and methods to study this degradation behaviour are discussed together with a summary of efforts to achieve the ideal thermogel with appropriate degradation profile.

7.2 Relevance of Thermogels

The favourable characteristics of thermogels make them ideal candidates for numerous applications that need to interface with biological tissue. With a surge in the ageing population in recent years, thermogels will prove to be a critical component in future advances in regenerative medicine. This field has been defined as: 'The repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including congenital defects, disease, trauma and ageing'.⁵ The importance of thermogels in the healthcare sector has been established in extensive studies involving drug delivery, cell delivery and tissue engineering.^{6–8} This includes areas such as ophthalmology, cardiac treatments, nerve therapy, drug delivery and tissue engineering (Figure 7.1). Recent advances have also been made in the use of thermogels for the 3D printing of organ components such as kidneys.^{9,10}

7.2.1 Drug Delivery

Drug delivery is arguably the most intensively researched application of thermogels. Scientists in this field have explored the use of many materials including poly(ϵ -caprolactone-*co*-glycolide), poly(ethylene glycol), poly(propylene

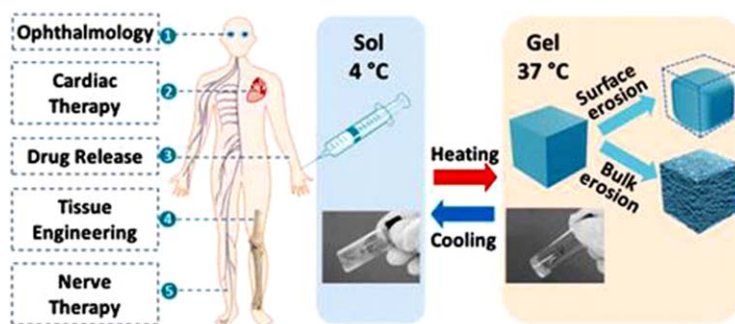


Figure 7.1 Application of thermogels in various biomedical areas.⁵¹
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glycol) and poly(lactide-*co*-glycolide) to synthesize drug-carrying thermogels.^{11–13} They have studied the release rates of various drugs and examined the factors that affect these release rates.^{11,12} Encapsulation of the drugs can be achieved by controlling the environment temperature, which is a simple process that does not involve any toxic chemicals. Poly(ϵ -caprolactone-*co*-glycolide)-poly(ethylene glycol)-poly(ϵ -caprolactone-*co*-glycolide) [P(CL-GL)-PEG-P(CL-GL)], for example, has a sol-to-gel transition at 37 °C, which matches the human body temperature.¹¹ This means that the gel can be injected and will gel *in situ*. A minimally invasive drug depot can be created with subsequent sustained release of drug.

7.2.2 Tissue Engineering

Tissue engineering is an integrated discipline that combines the use of scaffolds, cells and growth factors to repair, replace or augment the functions of injured or diseased tissue. It has emerged as the preferred method over the use of autografts, allografts or xenografts. An autograft is retrieved from one body part of the patient and transplanted into another part of the body. Allografts and xenografts are grafts retrieved from donors of the same species and another species, respectively. Although the use of autografts minimises the possibility of disease transmission and tissue rejection, which are the common issues faced from the use of allografts and xenografts, it faces the problem of function loss at the donor site. Unlike these techniques, tissue engineering cultures cells extracted from the patient, which minimises the chance of rejection and prevents the possibility of contracting a disease from the donated graft. The only disadvantage is the length of time for the tissue regeneration, which might prolong the recovery time.

The scaffold is an important component of tissue engineering. It provides a platform for the cells to expand and grow, all the while serving as a protective barrier against the harsh environment. Developing a suitable scaffold, however, demands highly specific properties of the materials involved, such as biocompatibility, mechanical compatibility and biodegradability. Thermogels are one of the best candidates in this respect. The ability of thermogels to exhibit a physical change in response to thermal variation makes it possible for the scaffold to achieve *in situ* gelation. This property is extremely useful for tissue regeneration because the gel can be formed in an irregularly shaped defect site, allowing for high geometric compatibility.⁷ Moreover, it can be injected into difficult locations such as between vertebrae. This method of restoration is minimally invasive, and so considerable effort has been devoted to fine-tuning the delivery of cells/growth factors using this technique.

7.3 Importance of Degradability

Poly(*N*-isopropylacrylamide) is a commonly investigated component of thermogelling systems due to its LCST being close to body temperature.^{8,14}

However, this polymer has a non-degradable backbone, which is a problem for particular biomedical applications.⁸ Similarly, clinical investigations carried out in rabbits and mice indicate that the non-degradable nature of another common hydrogel component, the PEG-PPG-PEG triblock polymer, can lead to an increase in plasma cholesterol levels, causing hyperlipidemia.^{15,16} Therefore, it is important to remove implants made of this material from the body after it has served its function. The implant might be biocompatible initially but complications can surface after many years unless it can be resorbed into the body. If degradable, the implant can break down into safe products and be excreted from the body *via* the renal system. This eliminates the need for additional surgery to remove the implant.

7.4 Biodegradation

Biodegradation is the process of degradation of substances into smaller fragments that can eventually be integrated back into the environment by biological means. Before the enzymes can act on the polymers/gels, however, they have to be broken down into smaller pieces, which is achieved through surface or bulk erosion.

7.4.1 Surface Erosion

Surface erosion can be easily identified because the material shrinks over time. Although the gel exhibits the same degradation rate for any exposed surface, this does not necessarily mean that all the exposed polymer chains are degraded. The chains can be freed from the main structure and remain in the buffer. There are three main phases for this type of degradation. The first phase is the incubation period which is determined by the extent of crosslinking. The second phase marks the period of erosion. During this period, the physical crosslinks are broken down and a constant mass loss can be detected. In the last phase, the mass loss is no longer constant and random scission takes over.¹² Given that the erosion rate is directly proportional to the exposed surface area, a zero-order release model can be used to approximate this process. To increase the lifespan of the material, parameters such as dimension and shape need to be considered along with its chemical properties.

Polymers with the tendency to undergo this type of erosion usually have poor water uptake ability, and are known as hydrophobic materials. Although these polymers restrict the diffusion of the water molecules into the deeper region of the matrix, they are still reactive to water molecules *via* their hydrolysable bonds. Examples are poly(*ortho* esters).¹⁷ While they are hydrophobic, they possess pH-sensitive linkages that degrade under acidic conditions.¹⁸ At physiological pH 7.4, the hydrolysis process is slow, but it accelerates when the pH is lowered. Heller drew attention to an interesting finding for this type of system.¹⁸ He demonstrated that the addition of an excipient into a pH-sensitive system can influence the type of erosion it

experiences. For instance, the addition of calcium lactate, which is slightly acidic and has low water solubility, into a linear polymer of 3,9-bis(ethylidene 2,4,8,10-tetraoxaspiro[5,5] undecane, 1,6-hexanediol and *trans*-cyclohexanedimethanol led to more surface erosion. The reaction between the calcium lactate and the water molecules produced a more acidic environment and accelerated degradation. As the degradation rate only gained momentum in regions with water penetration, which was the outermost layer of the hydrophobic system, the system was more inclined to surface erosion. Since the outcome depends on the relative degradation rates between the inner and the outer regions, creating a more basic environment in the inner region should also lead to surface erosion. In the study, the poorly water soluble and basic salt magnesium hydroxide was introduced into the crosslinked polymer made up of 3,9-bis(ethylidene 2,4,8,10-tetraoxaspiro[5,5] undecane) and 2-methyl-1,4-butanediol. The results confirmed the hypothesis that stabilizing the inner region with a more basic environment can cause the system to lean towards surface erosion. In this latter technique, the degradation rate of the inner region was impeded by the basic environment. Degradation only began after the basic environment was neutralized by the influx of water molecules and the outflow of magnesium hydroxide. Hence, the erosion is limited to the surface.¹⁷

Another group of polymers that are susceptible to surface erosion are polyanhydrides.¹⁹ Poly[bis(*p*-carboxyphenoxy)methane] (PCPM) was used to evaluate the degradation behaviour of this polymer class. The tests were performed in 0.2 M sodium phosphate buffer at two different temperatures – 37 °C and 60 °C. The results obtained were typical surface erosion curves with an initial incubation period followed by a linear mass loss period (Figure 7.2). Visual observation further confirmed the degradation mechanism as the device grew smaller without the matrix collapsing. A drug release study was performed with cholic acid. The release profile coincided with the degradation curve of the polymer, which again confirmed that the system operated by the surface eroding mechanism.²⁰

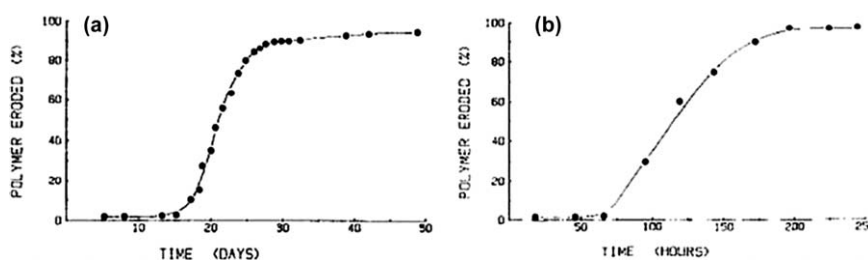


Figure 7.2 Erosion profiles of drug-free PCPM matrices at (a) 37 °C and (b) 60 °C. The matrices had dimensions of 0.24 cm² × 0.08 cm and 0.33 cm² × 0.05 cm and the tests were performed at 37 °C and 60 °C, respectively.²⁰ Reproduced from ref. 20 with permission from Elsevier, Copyright 1983.

7.4.2 Bulk Erosion

The terms degradation and erosion are critical to understanding the process of bulk erosion. Degradation is the reduction in molecular weight, while erosion is the loss of mass. Bulk erosion is characterized by a sigmoidal profile of mass loss. Mass is almost constant for the initial part of the curve and a rapid loss of mass is observed after a certain point of time. The length of the incubation period, marked by relatively constant mass, is determined by the time the polymer chains require to break down to a critical molecular weight that is water soluble. Although there is no significant mass loss in the beginning, this does not indicate that there is no degradation. Degradation begins the moment the polymer is exposed to the water/erosion medium.²¹ This sigmoidal profile may be due to the time the water molecules require to infiltrate the material followed by degradation throughout. Lack of mass loss is caused by the slower degradation rate on the surface of the material, which is holding the whole matrix together. Thus, there is minimal release of the degraded fragments into the medium. The sudden peak of release of the fragments into the medium is possibly due to the collapsing of the matrix. The rate of bulk erosion differs accordingly to the volume of the material and the molecular weight of the polymer. Generally, the rate of bulk erosion decreases with the volume of the material because there are fewer available bonds to be cleaved. Materials with free carboxylic acid end groups such as RG 504H and RG 502H degrade faster compared with their esterified counterparts.²¹ Unlike surface erosion, bulk erosion occurs throughout the matrix. Hence, the lifespan of a material undergoing bulk erosion is only affected by the chemical properties of the material. Aliphatic polyester such as poly(glycolic acid-co-DL-lactic acid), for example, experience bulk erosion because of susceptibility of the ester backbone to hydrolysis.²²

The mechanism of erosion depends on the rate of polymer hydrolysis, the diffusion rate of the water molecules and the matrix size. If the water molecules can infiltrate the matrix faster than the degradation of the material, bulk erosion will occur before significant surface erosion can take place. In contrast, surface erosion will result if degradation is faster than penetration by the water molecules. Hence, it is possible to manipulate the type of erosion that the material experiences. A faster diffusion rate can be achieved through blending with a more hydrophilic polymer. Alternatively, the material can be adjusted to undergo bulk erosion by reducing the size of the material. Decreasing the size of the material shortens the time required for the water molecules to reach the centre and hence, increases the rate of degradation through bulk erosion. Various models have been developed to predict the type of erosion.²³ For example, there is the model developed to gauge the 'erosion number' of the polymer matrix, and this dimensionless value can be used to determine the L_{critical} . A matrix larger than L_{critical} will likely experience surface erosion and a smaller volume will undergo bulk erosion.²⁴

7.4.3 Enzymatic Degradation

Enzymatic degradation relies on enzymes to cleave the backbone of the polymer. This type of degradation can be made very specific to certain enzymes. For example, a thermogel prepared with poly(alanine-co-leucine)-poloxamer-poly(alanine-co-leucine) was broken down *in situ* by the proteolytic enzymes elastases and matrix metalloproteinases. No degradation was observed with chymotrypsin, cathepsin B or cathepsin C.²⁵ This inherent specificity of this system is an advantage relative to other systems that depend on aqueous hydrolysis. The degradation rate can be varied by regulating the number of sites the enzymes can act upon. In addition, the efficacy of the system can be more tightly controlled by encapsulating the enzymes within the gels made of biodegradable polymer.

There are various factors that can affect the degradation rate of the polymers, one of which is the polymer structure. For example, the stereochemistry of the polymer bonds affects the degradation efficiency. Li and coworkers compared the enzymatic degradation of three stereocopolymers. PLA₅₀-mes was found to degrade faster than PLA_{62.5}, which degraded faster than PLA₅₀-rac. This difference in degradation rate indicates that the proteinase K has a cleavage preference for LD, DL and LL bonds over DD bonds.²⁶ In another independent investigation of the differences among the enantiomers of fungicide triadimenol, the same observation was noted with *RS* and *SS* enantiomers degrading at an accelerated rate relative to *SR* and *RR* enantiomers, respectively.²⁷

Besides chirality, Li and McCarthy showed that the crystallinity of the polymers could determine the degradation rate. They studied the degradation of poly(L-lactide) (PLA₁₀₀) and poly (DL-lactide) (PLA₅₀) in the presence of proteinase K. The results indicated a declining weight loss rate in the order PLA₁₀₀₋₀ > PLA₁₀₀₋₁ > PLA₁₀₀₋₂ > PLA₁₀₀₋₃ > PLA₁₀₀₋₆₀. The degradation rates of PLA with varying crystallinities are summarized in Table 7.1. It appears that the more amorphous structures experienced accelerated weight loss relative to the more crystalline materials. However, an insignificant difference in the degradation rate was observed for PLA samples of low crystallinity.²⁸ These findings could be explained by considering the accessibility of the polymer chains. An orderly, structured polymer is difficult for the enzymes to access than polymer chains of a randomly organized polymer. This observation further implies that tuning of the degradation rate could be achieved by manipulating the number of repeating units, because

Table 7.1 Crystallinity and degradation rate.²⁸

Polymer	PLA ₅₀	PLA ₁₀₀₋₀	PLA ₁₀₀₋₁	PLA ₁₀₀₋₂	PLA ₁₀₀₋₃	PLA ₁₀₀₋₆₀
Crystallinity ^a (%)	0	6.4	13.3	25.7	32.1	49.2
Degradation rate ($\mu\text{g mm}^{-2} \text{ h}$)	1.9	2.2	2.1	2.1	1.6	0.3

^aDeduced from DSC thermograms.

fewer long repeating units tend to result in a more crystalline region than numerous short repeating units.²⁹

The chemical composition of the polymer is obviously a decisive factor in determining the rate of degradation. Wang and coworkers, for example, synthesised poly(lactide-*co*-glycolide) (PLGA) copolymers of two different molar ratios – 70/30 and 50/50. These copolymers were then exposed to trypsin under physiological conditions. PLGA (50/50) was found to degrade faster than PLGA (70/30) with complete dissolution in about 6 weeks, whereas PLGA (70/30) required around 14 weeks. Similar results were obtained for the weight average molecular weights (Figure 7.3).³⁰ This finding highlights the observation that a greater glycolide content promoted faster degradation. This could be related to the increase in hydrophilicity that accompanies increased glycolide content. Glycolide is more hydrophilic than the lactic unit and thus better able to facilitate water uptake ester bond hydrolysis.

While the other factors are also important for obtaining the ideal degradation rate, the most crucial factor is the degree of flexibility of the polymer chain. Without flexibility, the polymer cannot fit into the active site of the enzyme.

The concentration of enzyme also determines the activity at that instant. Moreover, enzymes are very sensitive to their environment. They have a range of pH and temperatures over which they can work efficiently. Any deviation from that optimal range will cause a decline in activity. For example, neutralization of the stratum corneum results in an abnormally high activity of the serine protease, causing degradation of the lipid processing enzymes and the corneodesmosome proteins.³¹ Hence, deviation from the optimized pH range can change not only enzyme activity but also cause the enzymes to lose their specificity and, in turn, their ability to degrade the intended substrates. Likewise, a deviation from their optimal temperature can affect their capacity to break down substrates. Lowering the incubation temperature results in correspondingly lower digestive enzyme activity.³²

7.5 *In Vivo* Degradation

It is difficult to truly simulate *in vivo* degradation, notwithstanding the numerous models that have been developed. Various studies have reported that degradation experienced *in vivo* is faster than that observed *in vitro*.^{33,34} There are many possible factors to account for this. After all, *in vivo* conditions are very different from the contrived *in vitro* environment. The interaction between a material and its environment is more complicated *in vivo*, with the presence of proteins in the blood plasma and the different viscosity of blood compared with phosphate buffers commonly used in *in vitro* experimentation. For example, a comparative study demonstrated that the M_n of poly(trimethylene carbonate-*co*-D,L-lactic acid) copolymer decreased from 89 567 kDa to 17 242 kDa after 4 weeks of implantation,

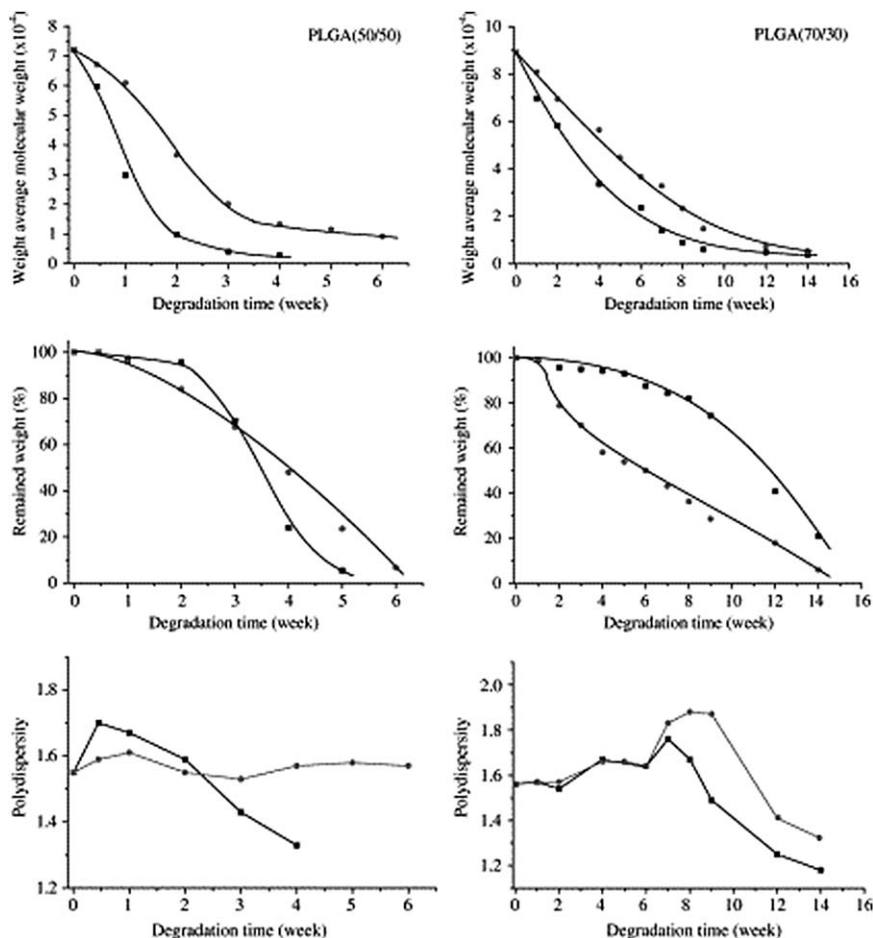


Figure 7.3 Degradation behaviours of porous PLGA (50/50) and PLGA (70/30) foams under physiological conditions. (■) and (●) represent with trypsin and without trypsin, respectively.³⁰ Reproduced from ref. 30 with permission from Elsevier, Copyright 2003.

whereas the M_n for the same polymer treated with phosphate buffer *in vitro* decreased to 39 864 kDa after 4 weeks. The reasons for the accelerated degradation *in vivo* were the synergistic effects of enzymes and phagocytes in addition to hydrolysis.³⁵ The importance of the environmental conditions was further emphasized in another study in which the different degradation rate *in vivo* was associated with the presence of particular organic compounds.³⁶ It is also imperative to note that the factors responsible for differences in degradation rates for different individuals depends on their health, activities, diets and habits. While researchers can attempt to mimic *in vivo* conditions by subjecting the thermogel to physiological conditions of pH 7.4 and 37 °C, it is not possible to emulate each patient's physiology.

7.6 Factors Affecting the Degradation Rate

The rate of degradation is important to particular thermogel applications. As a drug carrier, for example, the degradation rate, which is closely tied to the drug release rate, must be strictly controlled. A higher release rate can result in toxicity, whereas a slower release may render the treatment ineffective. When used as an implant to augment or repair a defect site, the thermogel's role is to either replace or support the injured body tissue. It is therefore important for the implant to degrade at the same rate as the tissue that is being regenerated to provide maximum mechanical support for the cells to grow and protect the cells from the harsh environment. To achieve better control of the degradation rate, it is necessary to understand the factors that influence it.

7.6.1 Material Properties

The constituents of the material are the main factors that will determine the degradation rate. Table 7.2 shows a list of polyesters and their degradation rates. The time for complete degradation ranges from as short as 6 weeks to as long as a few years.

7.6.2 Packing of Micelles

Each implant or drug carrier usually consists of mixed materials to fulfil the requirements of mechanical strength and biostability. An example is a thermogel made up of poly[(*R*)-3-hydroxybutyrate] (PHB), poly(propylene glycol) (PPG) and poly(ethylene glycol) (PEG). In this system, the packing of the micelles is the primary parameter that determines the degradation rate.

Table 7.2 Degradation rates for the different polyesters.

Polymers	Time for complete degradation (weeks)	Mass loss (%)	Conditions	References
Poly(lactic acid) (PLA)	>2	12–14	<i>In vivo</i> (rat)	37
Poly(glycolic acid) (PGA)	10–12	100	0.2 M citrate-phosphate buffer (pH 7 and 37 °C)	38
Polycaprolactone (PCL)	6	100	5 M sodium hydroxide (37 °C)	39
	>240	18	Phosphate buffered saline (pH 7.4 and 37 °C)	
Poly(hydroxybutyrate) (PHB)	>6	Insignificant loss	<i>In vivo</i> (mice)	40

Higher PHB content disrupts the packing of the micelles and leads to faster degradation.¹² Higher hydrophilicity leads to looser and larger micelles. In another study, Kim and coworkers developed a thermogel using poly(ethylene glycol-*b*-(DL-lactic acid-*co*-glycolic acid)-*b*-ethylene glycol), or PEG-PLGA-PEG. This thermogel was shown to be more stable than a thermogel made of Pluronics. The PEG-PLGA-PEG gel did not dissolve upon dilution and lasted 1 month, whereas poly(ethylene oxide), poly(propylene oxide), poly(ethylene oxide) (PEO-PPO-PEO) eroded within 1 day. The difference was attributed to the micelle packing. For Pluronics, the micelles formed at an increased temperature. At high temperature, a new intermixed segment was introduced between the PLGA core and the PEG corona. These researchers hypothesised that strong micelle packing was achieved by the increased interphase volume and miscibility. PEG chain could have interpenetrated the micelles, causing aggregation of the hydrophobic segments in the intermixed region, resulting in stronger micelle packing.⁴¹

7.6.3 Bond Type

The type of bonding also affects its degradation rate. The triblock PEO-PPO-PEO has a relatively slow degradation rate, which can be attributed to the strong ether bonds. By contrast, the ester bonds of PEG-PLGA-PEG are more easily hydrolysed, and this triblock polymer degrades more quickly than its polyether counterpart.⁴²

7.6.4 Ratio of Hydrophilic to Hydrophobic Sections

The hydrophobicity of the polymer can greatly influence the eventual degradation rate. The degradation rates of copolymers consisting of hydrophilic and hydrophobic sections as measured by weight loss over specific periods are listed in Table 7.3. The trend derived from the table is that an increased proportion of hydrophilic to hydrophobic sections is associated with greater weight loss. A possible explanation for this observation could be that the hydrophilic section promotes diffusion of free polymer chains away from the matrix, and hence a more visible weight loss is detected. Therefore, an increased portion of hydrophilic polymer can be used to accelerate the degradation rate of the system while more hydrophobic polymers can be employed to achieve a slower degradation rate.

7.6.5 Number of Sites for Enzymatic Action

The number of functional groups that can be enzymatically hydrolysed also determines the degradation rate. Likewise, there are circumstances when the concentration of enzyme is the limiting factor.

Table 7.3 List of copolymers and their weight losses suggests that increased hydrophilicity accelerates weight losses.

Copolymers	Ratio	Weight loss (%)	Conditions	References
Poly(DL-lactide)/ lactide/10K PLGA (LA/ GA = 50/50)	50 : 10 : 40	40	6 weeks	43
	60 : 10 : 30	15–20	pH 7	
	70 : 10 : 20	15–20	37 °C	
	90 : 0 : 10	10	Phosphate buffered saline	
Caprolactone/ lactide	100 : 0	0	4 weeks	44
	87 : 13	0	pH 7.6	
	51 : 49	5–10	45 °C	
	32 : 68	5–10	0.1 M phosphate buffer solution	
Pluronic DL- lactide/ε- caprolactone	0 : 1	Insignificant	60 weeks	45
	1.4 : 1	43–50	pH 7.4	
			37 °C	
DL-lactide/ glycolide			0.13 M isoosmolar phosphate buffer	46
	100 : 0	30–40	80 days	
	75 : 25	30–40	37 °C	
	50 : 50	80–90		
PHB/PEG	39 : 61	7	14 weeks	47
	32 : 68	9	pH 7.4	
	25 : 75	10	37 °C	
	14 : 86	13	Buffer solution	

7.7 Techniques to Study the Degradable Behaviour of Thermogels

7.7.1 Mass Loss

The rate of mass loss is used to study the degradation behaviour of gels (Figure 7.4). In an investigation to examine the impact of increasing thiol groups on network structure, Bowman and coworkers studied the degradation of thiol-acrylate gel. These gels were subjected to a stimulated *in vivo* environment at approximately physiological conditions. They were incubated in an orbital shaker operating at 60 rpm to simulate the condition of movement. The mass loss was plotted against time (Figure 7.5).⁴⁸ The rate of degradation could be derived from the slope of the curve. The steeper the curve, the faster the degradation rate. The duration of time that the gel takes to reach 100% mass loss was used as an indication of its stability.

7.7.2 Molecular Weight Comparison

The molecular weights of degraded fragments contain crucial information for our understanding of degradation behaviour. Gel permeation chromatography (GPC) is the most common tool for this purpose (Figure 7.6).

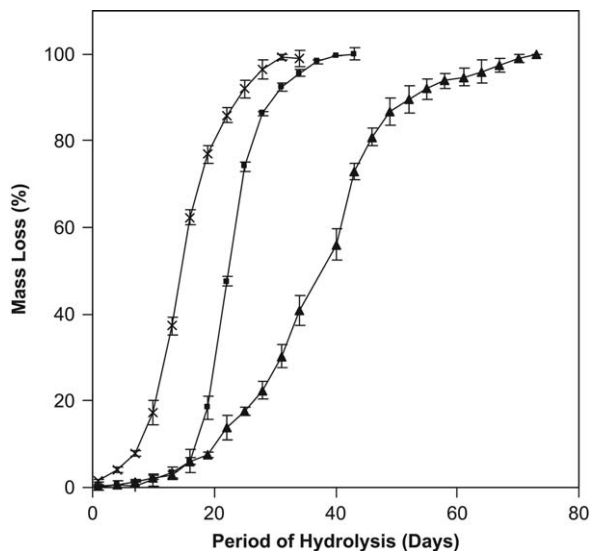


Figure 7.4 Mass loss (%) of poly[(*R*)-3-hydroxybutyrate] (PHB), poly(propylene glycol) (PPG), and poly(ethylene glycol) (PEG)-based EPH hydrogel, poly(PEG/PPG/PHB urethane) after incubation in PBS at pH 7.4 and 37 °C (▲: EPH(2%), ■: EPH(5%), ×: EPH(8%)).¹² Reproduced from ref. 12 with permission from Elsevier, Copyright 2007.

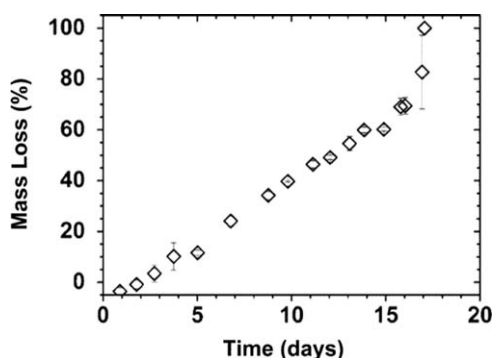


Figure 7.5 Plot of mass loss vs. time of a thio-acrylate network (50 wt% monomer mixture in DMSO) incubated under physiological conditions and subjected to shaking at 60 rpm.⁴⁸ Reproduced from ref. 12 with permission from Elsevier, Copyright 2007.

The larger fragments elute faster than their smaller counterparts because smaller fragments can take a longer path between the silica beads of the GPC column and hence take a longer time to be eluted. Parameters such as the number average molecular weight, polydispersity index, weight average molecular weight and viscosity molecular weight can be derived from GPC data. The kinetics of degradation can be determined. For instance,

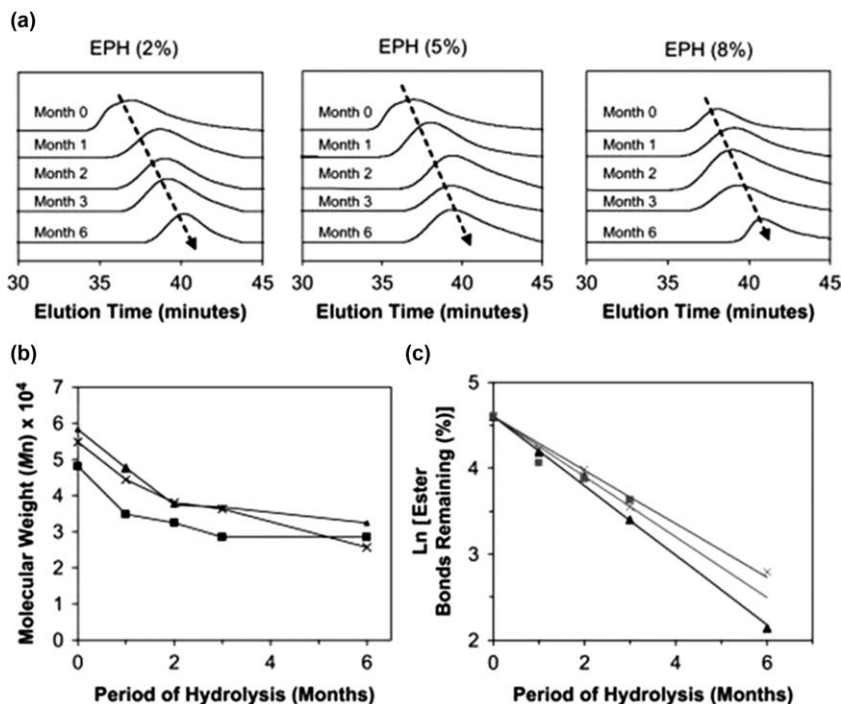


Figure 7.6 (a) GPC profiles of the copolymer degradation products in chloroform extracts from the PBS buffer at various hydrolysis periods. (b) Changes in molecular weight of the copolymer degradation products with time (▲: EPH(2%), ■: EPH(5%), ×: EPH(8%)). (c) Plot of the natural logarithm of the fractional ester bonds remaining against degradation time of the polymers (▲: EPH(2%), ■: EPH(5%), ×: EPH(8%)).¹² Reproduced from ref. 12 with permission from Elsevier, Copyright 2007.

Loh *et al.* found that ester bond disintegration obeyed a pseudo-first order rate law:

$$\frac{-d[Es]}{dt} = kEs$$

where k is the pseudo-first-order rate constant and Es is the fractional ester bonds (%) remaining at time t . Such a model is useful for characterizing the degradation rate and the amounts of ester bonds remaining at any time t .¹²

7.7.3 Surface Topography (Scanning Electron Microscopy)

The degradation behaviour of gels can also be analysed visually using a scanning electron microscope (Figure 7.7). Scanning electron microscopy (SEM) works by detecting the secondary electrons that are given off during the interaction between high energy electrons and atoms on the surface of

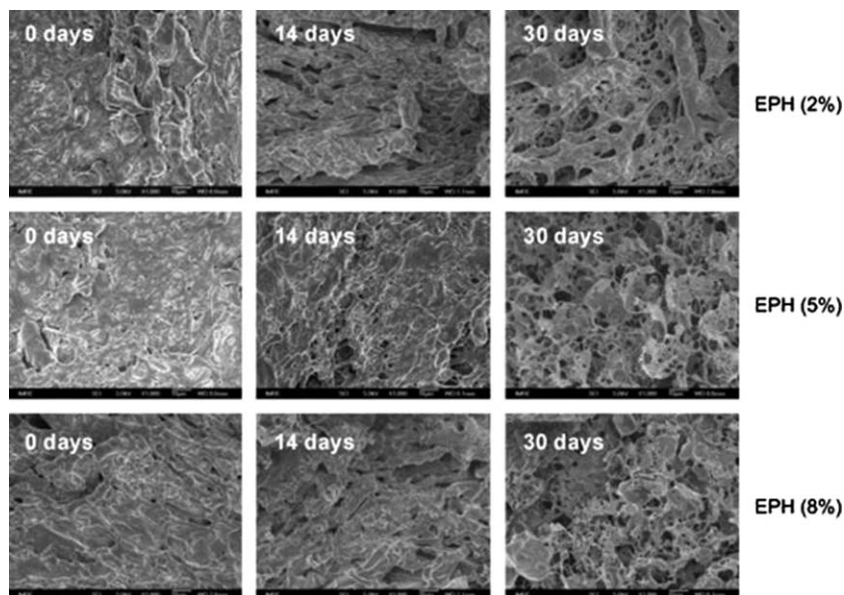


Figure 7.7 SEM images of hydrogel residues after various periods of degradation under physiological condition (PBS at pH 7.4 and 37 °C).¹²
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the lyophilized gel. Differences in surface topographies can be related to the degradation of the gel. An example reported by Chen and coworkers investigated the morphological changes of highly porous chitosan- α , β -glycerophosphate hydrogels upon exposure to 3 h of 0.25% (w/v) trypsin degradation at 37 °C. It was found that the structures were different after the enzymatic degradation and the pores were enlarged (Figure 7.8).⁴⁹

7.7.4 Fourier-transform Infrared Spectroscopy

Another technique to study gel degradation behaviour is to compare the infrared (IR) absorption peaks of gel residues at various time points by Fourier-transform infrared spectroscopy (FTIR). Changes in absorption peaks will be indicative of a change in the property of the gel, and thus of degradation. The IR absorption by PHB ester carbonyl bonds, for example, became broader, reduced in size and were red shifted over time in buffer under physiological conditions (Figure 7.9).¹²

7.7.5 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy makes use of the interaction between radio-frequency radiation and spin-active nuclei (^1H , ^{13}C , ^{19}F , ^{31}P , etc.) in a strong magnetic field. Changes in local geometry caused by degradation manifest in a change in local magnetic field that will be

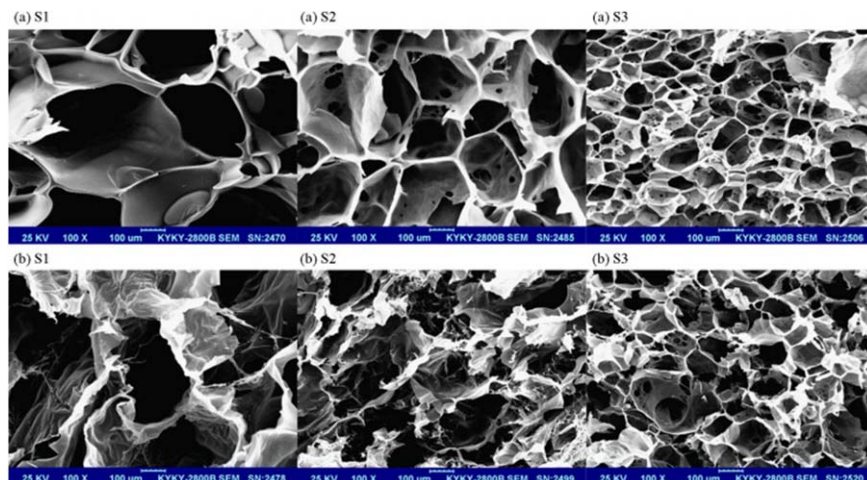


Figure 7.8 SEM images of chitosan- α,β -glycerophosphate hydrogels (a) before exposure to 0.25% (w/v) trypsin degradation and (b) after 3 hours' incubation with trypsin at 37 °C.⁴⁹

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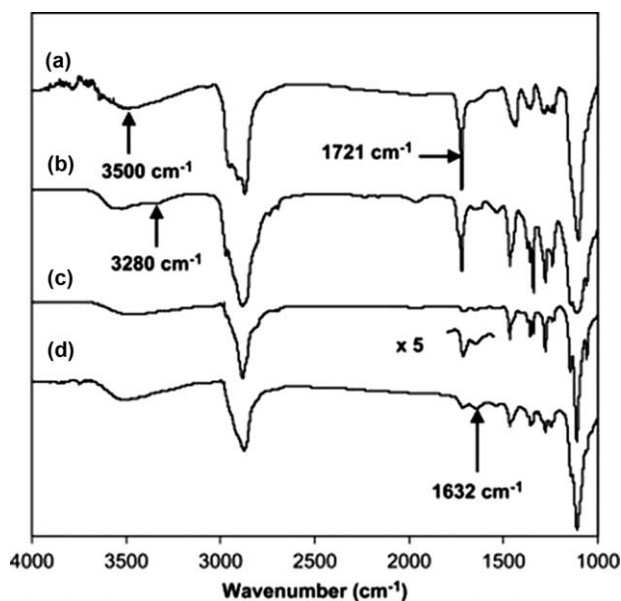


Figure 7.9 FTIR spectra of the poly(PEG/PPG/PHB urethane)s (5%) samples after degradation under physiological conditions (PBS at pH 7.4 and 37 °C). (a) Poly(PEG/PPG/PHB urethane)s (5%) control at day 0, (b) gel residue after degrading for a month, (c) water-soluble fraction after a month of degradation, (d) water-soluble fraction that had undergone 6 months of degradation.¹³

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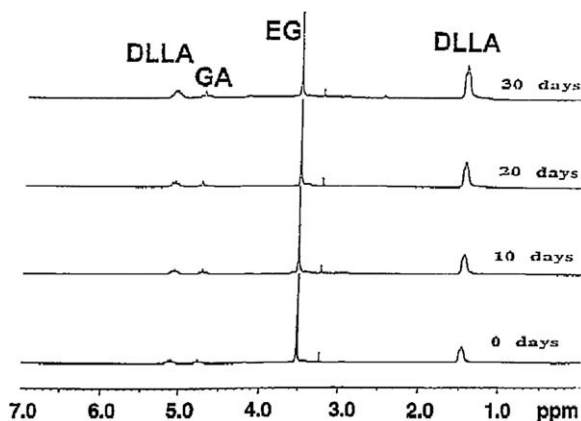


Figure 7.10 ^1H -NMR spectra demonstrating the change in a PEG-PLGA-PEG triblock copolymer gel in chloroform- d over 30 days of degradation (GA, DLLA and EG represent glycolic acid, DL-lactic acid and ethylene glycol, respectively.)¹²
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observed as a change in chemical shift. ^1H -NMR spectroscopy has been used to monitor the breakdown of PEG-PLGA-PEG triblock copolymer gels in chloroform- d over 30 days (Figure 7.10). The peak that corresponded to DL-lactic acid (DLLA) at approximately 1.5 ppm was noted to increase with respect to the peak that belonged to ethylene glycol (EG) at around 3.5 ppm. This change in the height of the peaks was used as an indication of the degradation occurring over the span of 30 days.¹³ An advantage of this technique is the possibility of identifying short fragments of the degraded products.

7.7.6 Technique Comparison

Table 7.4 summarizes the advantages and disadvantages of the various techniques used by researchers to identify and quantify degradation. There are different types of data available, including the change in the polymer chains, the kinetics of the system, the stability of the system and the surface morphology. To fully understand the degradation mechanism of a system, it is necessary to employ more than one technique. A single test, as shown in the following table, is unable to provide all the data that is required to understand the degradation mechanism.

7.8 Future Perspective

Recent years have seen the expansion of potential biomedical applications for degradable thermogels, ranging from carriers for drugs and scaffolds for tissue engineering to cell carriers and chemotherapy for treating cancer.

Table 7.4 Comparison of the various methods used to detect degradation.

Techniques	Advantages	Disadvantages	References
Mass loss	– Data on kinetic and stability of the system can be obtained.	– The change caused by degradation is unknown.	48
Molecular weight comparison (GPC)	– Provides details about the change in the polymer chains. Data available includes number average molecular weight, polydispersity index, weight average molecular weight and viscosity molecular weight.	– Only indicates if degradation has occurred but no details on the change in structure is available.	12
Scanning electron microscopy	– Provides visual illustration of the changes in surface morphology.	– Only indicates if degradation has occurred but no information on the change in polymer chain is available.	49
Fourier-transform infrared spectroscopy (FTIR)	– Information on the bonds which are forged or cleaved.	– Degradation kinetics unknown.	12
NMR spectroscopy	– The change in chemical structure can be identified. – Short fragments of degraded products can be measured.	– Degradation kinetics unknown.	13

A recent study focused on loading irinotecan, an antitumour drug, into a thermogel and demonstrated that the drug-loaded gel was effective in reducing the size of SW620 human colon tumours.⁵⁰ While there remained a mild side effect, it demonstrates the potential of this technology to be used for tumour treatment.

Given their unique properties, it is anticipated that degradable thermogels will be an important tool for medical treatments and regenerative medicine. To fully unleash this potential, more research is required to enable customization of the gel for particular applications. Thermogel degradation rate, mechanical strength, gelation speed, biocompatibility and immunogenicity are all parameters that need to be tuned for drug-release or tissue-engineering applications. There is a wide variety of materials available to biomedical engineers, with many yet to be explored. The control of these physical, chemical and biological parameters will be an optimization problem to be solved for each treatment situation.

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CHAPTER 8

From Bench to Bedside – OncoGel™, an In Situ Hydrogel for In Vivo Applications

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8.1 Introduction

The need for drug delivery systems to improve safety, efficacy and patient compliance is well documented. There exist various drug delivery systems, including microspheres and nano particles, as well as stimuli responsive systems.^{1–3} Thermosensitive polymers are a subset of stimuli sensitive polymers which undergo a phase transition (sol to gel) when subjected to a change in temperature. Regel™ is a biocompatible and biodegradable ABA tri-block polymer that exhibits reversible thermal gelation.⁴ It consists of poly(lactide-co-glycolide) and poly(ethylene glycol) arranged in an ABA or BAB sequence, with a defined molecular weight and hydrophobic/hydrophilic balance. Modification in the hydrophobic:hydrophilic ratio of the polymer constructs results in Regel™ being water soluble below room temperature and a gel at body temperature.⁴ As Regel™ is a physically formed

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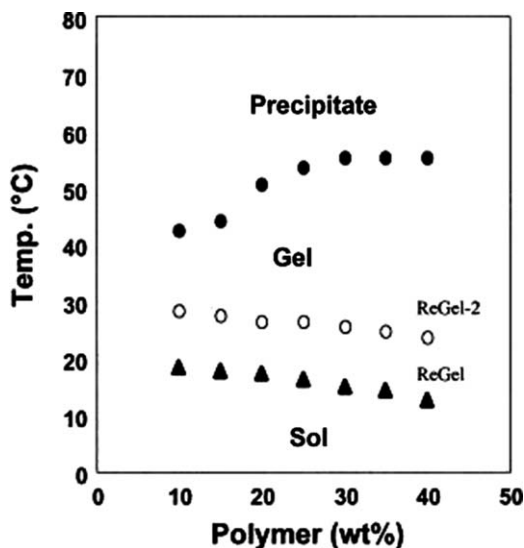


Figure 8.1 ReGel phase diagram.
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hydrogel, the sol-to-gel transition occurs within seconds, without any chemical modification of the constituent co-polymers.⁵ This reversible gelation behavior at physiologically relevant temperature (37 °C) is illustrated in the phase diagram (Figure 8.1).

OncoGel™ (ReGel™/paclitaxel) is a formulation of the chemotherapeutic, intratumorally injectable drug paclitaxel, developed by MacroMed Inc. (Salt Lake City, Utah, USA) for local treatment of solid tumors.⁵ Paclitaxel is a tubulin-binding agent, causing mitotic arrest and apoptosis. ReGel™ increases the solubility, stability and sustained release of hydrophobic drugs such as paclitaxel, and the ReGel™/paclitaxel system can achieve sustained drug release for about 6 weeks. OncoGel™ combines controlled release with physical targeting of the tumor site, either *via* intralesional injection or direct placement into the tumor cavity after resection.^{6–8} An advantage of this therapeutic regime over the systemic administration of paclitaxel is that it provides continuous release of the therapeutic agent throughout the tumor, irrespective of its vascular status. Moreover, OncoGel™ exhibits minimal toxicity and so can be used as a component of combination chemotherapies and surgery. Similarly, paclitaxel's anti-neoplastic and radiosensitization properties can be exploited by combining OncoGel™ with radiotherapy.^{9,10}

8.2 Non-clinical Safety and Efficacy Evaluation

Several non-clinical studies have explored OncoGel™'s anti-cancer activity to evaluate its potential biomedical applications. These included establishing its safety relative to paclitaxel alone and in combination therapy.⁵

8.2.1 Safety Studies

Various studies have been conducted in rats, dogs and pigs to evaluate the safety of OncoGel™ when administered to skin (subcutaneous injection), central nervous system (CNS) tissue, and the pancreas..^{4,7,8,11,12} The focus of the studies in rats and dogs was to establish the no adverse event level (NOAEL) and the maximum tolerated dose level (MTD) in normal tissue. The outcome of these local tolerability studies determined that the dose-limiting toxicities (DLTs) were local in nature with no systemic toxicity at the starting dose.

8.2.2 Tissue Distribution Studies

After determining the DLT, it was necessary to carefully study the distribution of paclitaxel following release from the OncoGel™, in order to manage the potential additive toxicity. An adsorption, distribution, metabolism, and excretion (ADME) study of paclitaxel was performed following intralesional administration to a MDA-MB-231 breast tumor xenograft in mice. The study examined the distribution of radioactively labeled paclitaxel over a span of 42 days. Paclitaxel was reported to be localized within the tumor with minimal levels (<0.2%) detected in the blood, tissues or urine. The elimination route was *via* feces, similar to the paclitaxel elimination following systemic administration.⁴

8.3 Development of OncoGel™ as a Potential Cancer Therapeutic Drug

After acquiring the preliminary information related to the distribution and safety of OncoGel™, it was necessary to evaluate its efficacy in animal models. Novel drug delivery methods were explored for efficient delivery of OncoGel™ to the tumor site. The feasibility of combining OncoGel™ with chemotherapy, radiation therapy and surgeries was also explored.

8.3.1 Rat Model Studies

8.3.1.1 Spinal Cord

The spinal column is the most common site of skeletal osseous metastases, with lung and breast lesions being the primary metastasis sites.⁷ As advances are made in the diagnosis and treatment of the primary disease, and the life span of the patient increases, so does the need to treat symptomatic distant metastases. Local chemotherapy can potentially provide an option for the treatment of metastatic disease, and the efficacy of OncoGel™ in delaying paresis was tested on spinal metastases in a rat model.⁷ The pre-treatment hind limb function was evaluated using the Basso–Beattie–Bresnahan (BBB) locomotor rating scale. Animals were injected with

OncoGel™ 3.0% and OncoGel™ 6.0%, and their locomotive function was re-evaluated. All surviving animals demonstrated postoperative BBB locomotor scores of 21 in all limbs. On the 10th day, the average BBB scores for the control, OncoGel™ 3.0%, and OncoGel™ 6.0% animals were 9.00, 16.80, and 16.86, respectively. Although the histological analysis showed no evidence of toxicity to the spinal cord in any animal, the OncoGel™ 3.0% groups experienced transient decreases in hind limb motor function and required about 3 days to recover. The cause for this was attributed to the surgical technique employed during the intravertebral injections. Additionally, OncoGel™ was found to increase the life span of the rats. The median survival time for the control, OncoGel™ 3.0%, and OncoGel™ 6.0% animals was 14, 18, and 18 days, respectively. However, there was no significant difference in the size of the tumor mass between the control and treatment groups at the time of histological analysis. Nevertheless, the delay in the onset of paresis led to the possibility of OncoGel™ increasing the quality of a patient's life.

The efficacy of OncoGel™ in a combination therapy with surgery and radiotherapy was also evaluated in the spinal column metastases model. OncoGel™ was injected into the tumor cavity during surgery. Surgery alone delayed the onset of paresis, but surgery + OncoGel™ resulted in a higher median BBB score (21 *versus* 19, $P < 0.001$).⁸ OncoGel™ was also found to prolong the time to loss of ambulation by 20%. OncoGel™ was then used as an adjuvant to radiation therapy and further improved hind limb function was observed with an increased time for the loss of ambulation from 17 to 19 days. However, certain factors need to be considered when analyzing the results of this study. The efficacy of a single dose of OncoGel™ injection was determined *before* the onset of deficits by using *neurological assessment* and not direct animal imaging. This leaves scope for further clinical and laboratory studies comparing the effects of OncoGel™ after the onset of deficits in motor function. A follow-up investigation was conducted to evaluate the efficacy of the local delivery of paclitaxel in the treatment of intramedullary spinal cord tumor (IMST) in rats challenged with a lethal dose of intramedullary 9L gliosarcoma.¹³ The outcome indicated that OncoGel™ was safe for intramedullary injection in rats in doses up to 5 μl of 3.0 mg ml^{-1} of paclitaxel, but a dose of 5 μl of 6.0 mg ml^{-1} caused rapid deterioration in BBB scores. OncoGel™ at concentrations of 1.5 mg ml^{-1} and 3.0 mg ml^{-1} paclitaxel given on both Day 0 and Day 5 improved BBB scores and the median survival time relative to controls.

8.3.1.2 Glioma

Malignant gliomas are the most common type of primary brain tumor with relatively poor prognoses. The current treatment involves insertion of Gliadel® wafers for the sustained release of carmustine¹⁴ Cellular proliferation inhibitors like paclitaxel are known to be effective against gliomas, but treatment with this drug is hampered by poor penetration into the central

nervous system (CNS). The safety and potential synergistic effects of OncoGel™ in rats challenged with intracranial 9L glioma was demonstrated in a study which combined OncoGel™ with radiotherapy.¹⁵ OncoGel™ was intracranially implanted into 60 animals, divided into placebo (ReGel™), radiotherapy (XRT), OncoGel™ 6.0 mg m⁻¹ or OncoGel™ with a single dose of 20 Gy XRT. Animals treated with just ReGel™ showed no increase in survival rate when compared to the controls, but animals administered with OncoGel™ and XRT had a statistically significant increase in survival time when compared with XRT alone ($P=0.0182$). These results were supported by another report detailing the safety of 6.3 mg ml⁻¹ of paclitaxel for intracranial injection in rats.¹⁶ It was also established that combining OncoGel™ acts as a radiation sensitizer; thus, combining OncoGel™ treatment with radiation therapy is more effective than just the standalone treatment.

Combination therapies which synergistically target different pathways can improve the efficacy of cancer therapies. Temozolomide (TMZ) is an alkylating agent used to treat patients with glioblastoma.¹⁷ Paclitaxel is a mitotic inhibitor which is effective against glioma *in vitro*^{18,19} and sensitizes glioma cells to radiation therapy. TMZ and paclitaxel have different mechanisms of action and so it was hypothesized that the combination of TMZ (oral and local) and OncoGel™ might have a synergistic effect in a rodent model of gliosarcoma. Treatment with TMZ prolonged survival, and no signs of systemic or neurological toxicity were observed. The combination of OncoGel™ with oral or local TMZ resulted in 57 and 100% long-term survival, respectively, proving that the local delivery of TMZ was a better treatment option. Since most patients suffering from glioma undergo radiation therapy, the efficacy of OncoGel™ + TMZ + XRT was also explored. The improved therapeutic effect of a treatment regimen involving this triple combination was indicated by the statistically longer survival time ($P<0.0001$) compared with the combination of oral TMZ and XRT.²⁰ However, the poor correlation between animal models and the human response was recognized and an attempt was made to increase the effectiveness of a drug delivery device by using computational models combining fluid transport and mass transport submodels.¹⁴ This study attempted to bridge the gap between the rat model and human tissue by using a simplified model to compare the paclitaxel distribution in the two systems. The therapeutic penetration distance from the injection site was found to be 1–2 mm (Figure 8.2). Although the rat and human brains have a similar penetration distance, the fraction of brain tissue exposed to therapeutic concentration of paclitaxel was reported to be higher in rats than in humans.

However, simulations assuming sink conditions increase the effective therapeutic distances, and so it is inaccurate to assume sink conditions in brain tissue (Figure 8.2). Nevertheless, the study managed to establish that the penetration pattern of paclitaxel was similar to that of Gliadel® wafers, with paclitaxel maintaining effective concentrations for more than 30 days whereas Gliadel® wafers could do so for only 4 days.

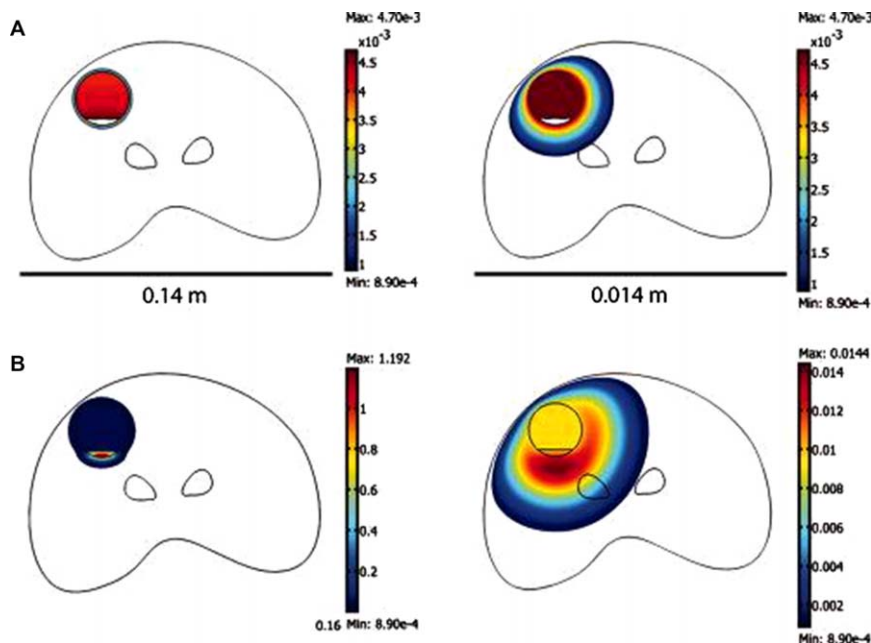


Figure 8.2 Comparison of paclitaxel distribution between human and rat brain 30 days after implantation. (A) Paclitaxel distribution in rat (right panel) and human (left panel) brain obtained when the solubility of paclitaxel in water is taken as the limiting factor for drug release from the polymer matrix from a 7% filled cavity. (B) Paclitaxel distribution in brain tissue assuming sink conditions in the brain from a 7% filled cavity. Based on the assumptions used in the simulation, drug penetration distances can differ by about one order of magnitude. Only concentrations above the minimum effective concentration have been displayed to visualize effective therapeutic distances. The convection term in the diffusion-reaction equation has been left out because the focus of this data is the amount of drug remaining in the polymer matrix due to the slow diffusion of paclitaxel and the difference in penetration scale between rat and human brains. Concentrations are given in mol m^{-3} .

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8.3.2 Pig Model Studies

8.3.2.1 Pancreatic Cancer

Patients diagnosed with pancreatic cancer often suffer from severe pain and biliary or intestinal obstruction. Chemotherapy and radiation are common treatments, but the prognosis is poor. With the development of better drug delivery systems, sustainable release of chemotherapeutic drugs is a possible treatment option. However, local delivery of drugs requires an easily accessible tumor. While superficial tumors of the skin, breast, and cervix provide an easy injection site, solid, deep-seated tumors require specialized

equipment for accurate placement of delivery systems such as OncoGel™. The feasibility of endoscopic ultrasound (EUS) for guiding injection of OncoGel™ was evaluated in a porcine model. OncoGel™ was successfully injected into the tail of the pancreas of a pig using an EUS-guided fine needle²¹ (Figure 8.3). The procedure was well tolerated and blood samples indicated no pancreatitis.

Gross and histological examination revealed a stable depot of OncoGel™ (Figure 8.4), with no report of extravasation out of the pancreas. The animals were euthanized after 14 days and showed localized fibrotic tissue changes and a decrease in inflammation.

A subsequent study reported that the concentration of paclitaxel varied with distance, *i.e.* with the high concentrations in the area around the depot and low concentrations 10 to 30 mm from the injection site.¹¹ This study used a similar EUS-guided injection of OncoGel™ and reported that the viscosity of the gel presented difficulties which could be overcome by using a

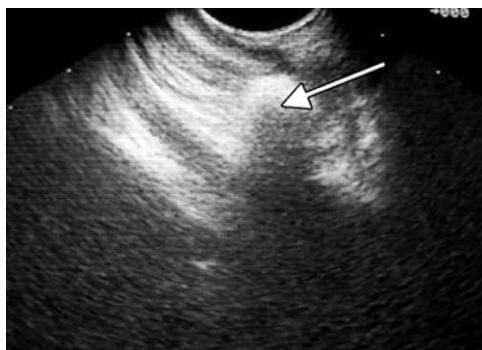


Figure 8.3 Endoscopic ultrasound image of the OncoGel™ injection site (arrow). Reproduced from ref. 21 with permission from Thieme, © Georg Thieme Verlag KG.



Figure 8.4 Macroscopic view of the OncoGel™ depot marked by India ink (arrow) in the third pig. Reproduced from ref. 21 with permission from Thieme, © Georg Thieme Verlag KG.

threaded syringe and pressure tubing between the syringe and the EUS needle to increase the pressure. The absence of pancreatitis and sclerotic or neurotic tissue formation indicated a good pancreatic tissue tolerability, but further investigation is required to understand the possibility of long-term complications such as bone marrow suppression, neuropathy or cardiotoxicity.

8.3.3 Human Clinical Trials

Pre-clinical studies of OncoGel™ established its cytotoxic potency and reported it to be localized within the injection site, thereby attenuating systemic toxicity. As the research progressed beyond animal model studies, phase one clinical trials were designed to evaluate the efficacy of OncoGel™ when administered intralesionally to superficially accessible solid tumor lesions in patients who had not undergone any other curative therapy.²² The blood chemistry and the hematology data was collected for 9 weeks following OncoGel™ injection. The tumor response was gauged from 3D images obtained from computed tomography (CT) scans, ultrasound and magnetic resonance imaging (MRI) and a caliper was used to obtain 2D measurements.⁵ The main objective of this study was to identify the MTD by observing the dose limiting toxicity (DLT) at various concentrations of OncoGel™. Sixteen patients received 0.06 to 2.0 mg paclitaxel cm⁻³ tumor volume in this trial. Although OncoGel™ placement into the tumor was well tolerated at doses up to 2.0 mg paclitaxel cm⁻³ tumor volume, eight patients had adverse local responses to OncoGel™ administration, such as injection site pain, muscle spasms and erythema. However, even at the highest dose of OncoGel™, no DLT was reported, confirming the animal model data regarding the localization of the OncoGel™ depot. Efficacy analysis was also performed using modified World Health Organization (WHO) criteria comparing the change of the tumor volume from baseline every 4 weeks. Six patients were reported to have a stable disease, and eight patients were classified as having progressive disease. However, the significance of these results remains questionable due to the small sample size and varied tumor types (breast, lymphoma, malignant melanoma, *etc*). Moreover, the enrolled patients might have been previously exposed to paclitaxel or other therapeutic agents, which could cause them to be less sensitive to OncoGel™. Thus, the results looked promising enough to continue with clinical studies to achieve more reliable results.

Tumors are generally treated using combination therapies such as chemotherapy, radiation and surgery. Paclitaxel could be used as an adjuvant to any of these as discussed in the non-clinical studies, but it was reported that 18–24 h incubation with paclitaxel was needed to sensitize the cancer cells to radiation.²³ Moreover, this sensitivity was only effective for a short period of time and declined rapidly after removal of paclitaxel.²⁴ OncoGel™ could provide sustained release of paclitaxel for a period of 6 weeks, increasing the efficacy of radiotherapy.

8.3.3.1 Esophageal Cancer

The annual number of deaths caused by esophageal cancer is estimated to be approximately 300 000.²⁵ Approximately 50% of diagnoses are made when the disease is in its final stages. OncoGel™ was recognized as a potential alternative to morbidity-inducing treatment options such as chemotherapy, radiotherapy and surgery. A multi-national US phase 2a dose escalation study was conducted to evaluate the efficacy of a dual therapy regimen (OncoGel™ + XRT) on patients suffering from advanced esophageal disease without having undergone chemotherapy. OncoGel™ of varying concentrations (1.5, 3.1 and 6.3 mg ml⁻¹) was successfully injected using linear EUS guidance. Patients were subjected to 28 fractions of 1.8 Gy radiation 3 days after the injection. No DLT was observed, and OncoGel™ did not add to the risks of XRT.²⁰ The pharmacokinetics of paclitaxel was not influenced by the presence of radiation. However, caution must be exercised when making the comparisons, since this study did not involve a cohort of patients undergoing XRT only.²⁰ The intratumoral concentration of OncoGel™ was 0.48, 1.0, and 2.0 mg paclitaxel per cm³ of tumor.^{6,12} Peak plasma concentrations were related to the OncoGel™ concentration and ranged from 0.53 to 2.73 ng ml⁻¹. OncoGel™ was well tolerated in the body; 82% of the patients ($n = 11$) were found to show an improvement in dysphagia over the study period. On a 5-point scale, 55% had a 2-point improvement and three had a 1-point improvement. The efficacy results were positive; two patients were reported to have a progressive response, whereas six patients were classified as having a stable disease and two had progressive disease. Figure 8.5 presents the image of exophytic tumor before and after the combination therapy. The biopsies collected at the end of week 11 were negative for four patients, and two patients with stage 3 disease showed significant improvements and were considered for resection.

The phase 2b study had tumor response as the primary endpoint and the safety, survival and pathological complete response (pCR) of the tumor were the secondary endpoints. A control group comprised of patients receiving standard of care (5-FU), cisplatin and XRT (chemo radiotherapy) while the treatment group consisted of patients with OncoGel™ and chemo radiotherapy. The combination of OncoGel™ plus chemo radiotherapy increased the number of adverse events, even though the therapy was well tolerated. The overall response of the OncoGel™ treatment group was 12.5%, while the standard of care group had a 20% response. A clearer differentiation between the two groups was provided by the pCR, with only 12.5% of patients in the control arm experiencing pCR relative to a 27.7% response in the control group.

It is well established that systemic administration of paclitaxel increases the efficacy of chemo radiotherapy, thus the lack of significant improvement shown in this combined study was attributed to the localized delivery of paclitaxel in the form of OncoGel™. Since OncoGel™ failed to show a significant improvement, it was terminated as a potential therapy for esophageal cancer in 2010.²⁶

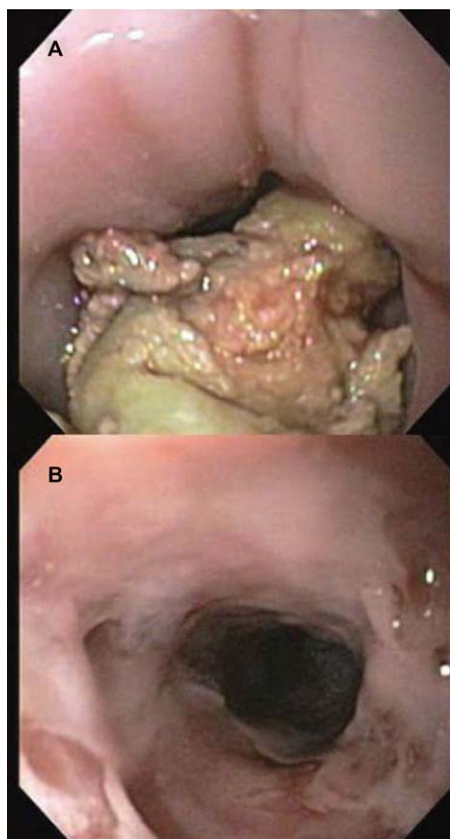


Figure 8.5 Image of an esophageal squamous cell carcinoma prior to (top) and following (bottom) administration of OncoGel™ and external beam radiation therapy (50.4 Gy). Reproduced from ref. 6 with permission from Wolters Kluwer Health, Inc.

8.4 Perspective

More than a decade of research has been dedicated to investigating OncoGel™ as an effective anti-cancer treatment regime for arresting metastasis and increasing patient compliance (Figure 8.6). It provided hope in this respect by exhibiting an excellent safety profile when administered to solid tumors. It showed minimal systemic toxicity and the DLT was observed to be local. It was also reported that OncoGel™'s intralesional injections ranging from 0.6 to 36 ml were well tolerated, with local pain being the only complaint. Most importantly, it provided a sustained release for over 6 weeks, which increased the tumor's exposure to the drug. Its efficacy as a combined therapy to surgery and radiation has been established in animal model studies and a phase 2a clinical study. However, the termination of OncoGel™ as a possible adjuvant to chemotherapy in esophageal cancer has been a

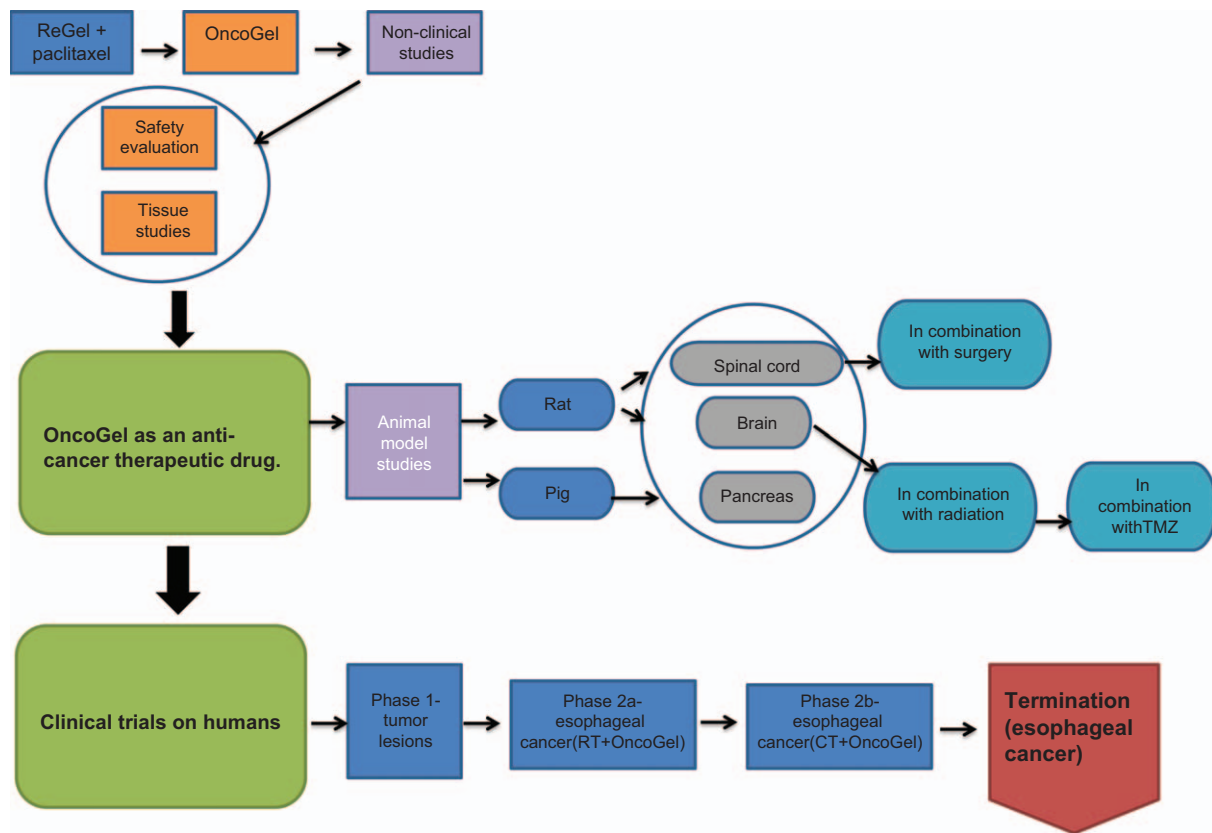


Figure 8.6 Developmental pathway of OncoGel™ towards clinical trials.

setback. There is a need, therefore, for further clinical studies focusing on other types of tumors. Treatment of brain tumors for which prognosis is poor could particularly benefit from the localized delivery of OncoGel™.

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CHAPTER 9

Hydrogel-based 3D Scaffolds for Stem Cell Culturing and Differentiation

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9.1 Introduction

Tissue engineering is a promising approach for treating damaged tissue or organs, without the need for donations from other sources. The most widely studied technique in this respect involves the use of a patient's own stem cells to grow functional cells to replace the damaged site. Stem cell growth and differentiation, however, requires an advanced biomimetic extracellular matrix (ECM) that can maintain structure in three dimensions.^{1,2} It is, of course, easier to grow cells in a 2D environment, for example, in a culture dish made of polystyrene. However, cellular growth in this unnatural shape can lead to unexpected phenotype changes and abnormal biomarker expressions. Human breast epithelial cells, for example, become tumor-like

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when they are cultured in 2D conditions but return to their original status upon culturing in a 3D environment.³ Chondrocytes become fibroblast-like in a 2D culture due to elevated type I collagen expression, while more type II collagen expression is observed in a 3D culture.⁴ It would appear, therefore, that cells cultured in a 3D scaffold are closer to a normal growth pattern. Understanding and guiding 3D cell culture is a key issue for tissue regeneration. Hydrogels are emerging as one of the most promising materials for the preparation of an artificial ECM.⁵ In contrast to natural systems, synthetic hydrogels are easier to control with respect to composition, conformation and degradability. More importantly, synthetic hydrogels are reproducible in manufacturing, and they are now being investigated for large-scale cell culturing and differentiation control.^{6–8}

Thermogels are well suited for encapsulating cells because of their mild sol-to-gel transition as temperature increases.^{9,10} Ideally, stem cells can be encapsulated in a non-biotoxic thermogel, which is liquid at a lower temperature and then is physically crosslinked in a more solid state when the temperature increases to body temperature. It is worth mentioning that the sol-to-gel transition process is mild and harmless for cells in contrast to chemical or photochemical crosslinking systems.⁸ Furthermore, as an added advantage for future body repair, the 3D scaffold thermogel can be injected at the target site using a conventional syringe, without the need for complicated surgery.¹¹ A thermogel matrix can fit any shape, even in irregular organ locations, and can encapsulate a cocktail of differentiating and growth factors. Moreover, hydrogels can provide some protection to cells from damaging free radicals, ultraviolet radiation and reactive chemicals, which might inhibit cell viability, proliferation or differentiation.⁶

Thermogels have been widely studied in the past two decades for their biomedical applications, including for drug delivery,^{7,12} tissue engineering,^{6,13} post-surgical adhesion prevention,^{14,15} embolization and wound dressing.⁸ Recently, researchers have also focused on controlling the growth and differentiation of stem cells by adding various inducing factors for growth and differentiation into the thermogel matrix, with some success. Jeong's group⁴ have reported a thermogel made of PA-PLX-PA block copolymer (Figure 9.1) that could encapsulate chondrocytes at low temperature, and form a 3D scaffold containing these cells at 37 °C. Copolymer L/D-PA-PLX-L/D-PA (PII) proved to be an outstanding 3D culture substrate for the growth and proliferation of chondrocytes, maintaining their spherical phenotype (Figure 9.1b).

Jeong's group¹² also investigated the effect of block copolymer concentration and hydrogel structure on the behavior of chondrocyte culturing. The viability of chondrocytes was excellent in 7.0–10.0 wt% PA-PLX-PA gel, with elevated type II collagen and sGAG expression (Figure 9.2a and b), indicating the importance of micromechanical cues such as the hydrogel's moduli and nanofiber thickness for 3D cell growth.

Han and Jeong groups¹³ have reported the utilization of a thermogel made of Arg-Gly-Asp (RGD) modified Pluronic F127 dimethacrylate (FM-RGD) polymer, as a 3D cell culture scaffold. This FM-RGD thermogel exhibited a

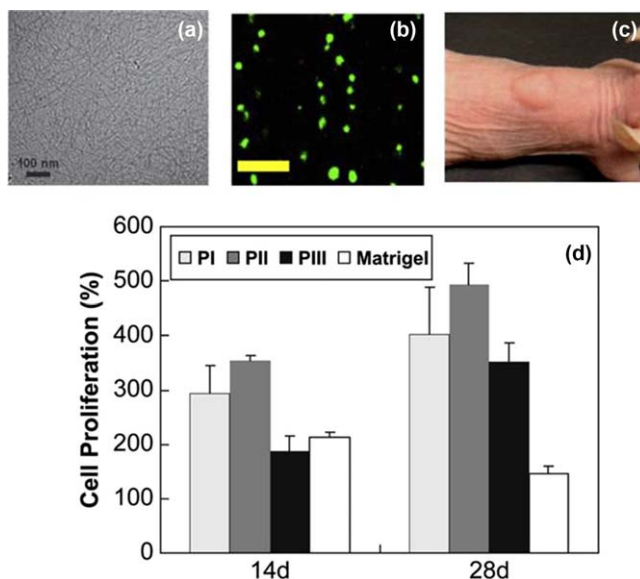


Figure 9.1 (a) Nanostructure of L/D-PA-PLX-L/D-PA. The scale bar is 100 nm. (b) Cell morphology in 28th day in L/D-PA-PLX-L/D-PA cultured systems. The scale bar is 200 μ m. (c) The formation of thermogels after the nude mice subcutaneous injection of the PII thermogel. (d) Chondrocyte proliferation rates within a thermogel 3D environment. Reproduced from ref. 4 with permission from Elsevier, Copyright 2010.

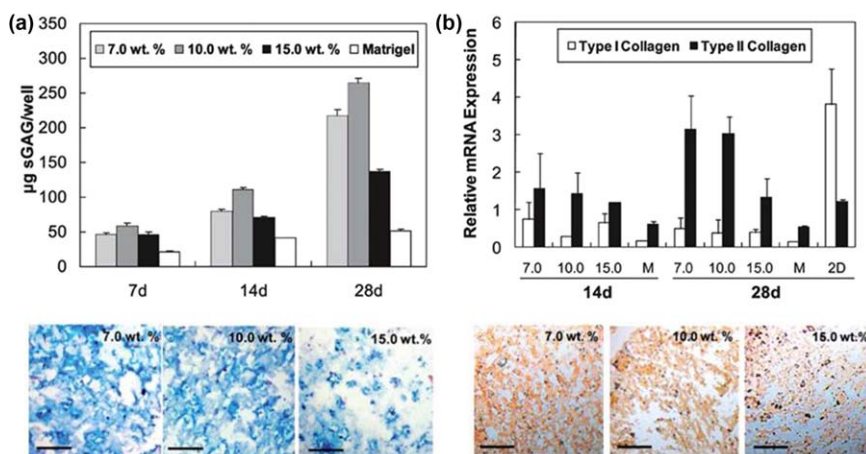


Figure 9.2 (a) sGAG and alcian blue stained image indicates chondrocytes in thermogels at different initial concentration of PA-PLX-PA. The scale bar represents 200 μ m. (b) Culturing chondrocytes in different 2D or 3D culture medium, the mRNA expression of type I and type II collagen was analyzed at the 14th and 28th days. M (Matrigel™) and 2D cultured system were also used for comparison. Type II collagen immunostaining image of chondrocytes at the 28th day. The scale bar is 200 μ m. Reproduced from ref. 12 with permission from The Royal Society of Chemistry.

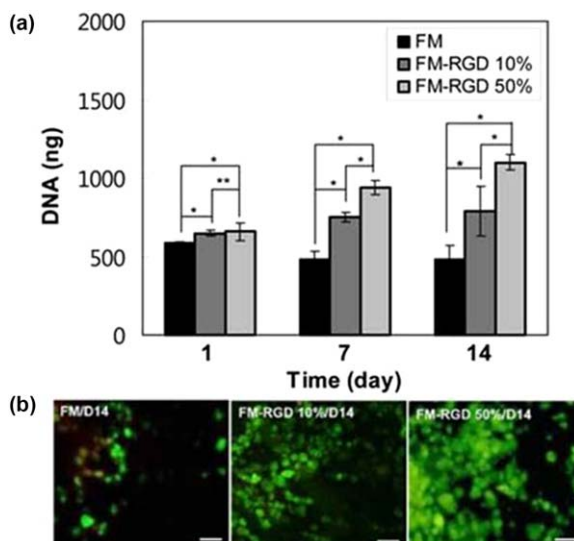


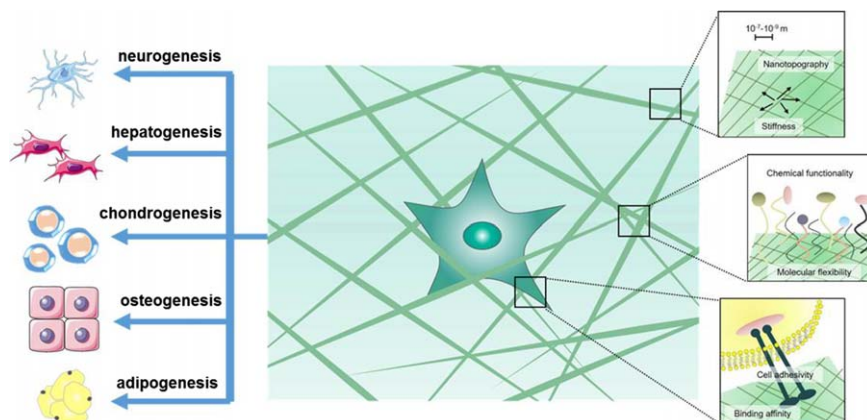
Figure 9.3 (a) Comparison of gene expression in thermogels of different RGD content. (b) Confocal microscopy images of cells in different RGD content thermogels at 14 days. The scale bar is 200 μm. Reproduced from ref. 13 with permission from Springer Nature, © The Polymer Society of Korea and Springer Netherlands 2012.

modulus of 8900 Pa at a culture temperature of 37 °C. FM-RGD showed significant improvement in cell viability, cell proliferation, gene expression and longer maintenance time for spherical phenotypes of chondrocytes than did the corresponding F127 dimethacrylate (FM) thermogel without RGD. Increasing RGD content from 10% to 50% led to chondrocyte proliferation and gene expression improvements (Figure 9.3a and b). This study highlighted the importance of a thermogel's chemical composition in the design of an artificial 3D ECM.

9.2 Hydrogel-based 3D Culturing and Differentiation of Stem Cells

Stem cells with differentiation ability are considered the next generation of therapeutic tools.^{14,15} Mesenchymal stem cells (MSCs) have important characteristics of immunosuppression, self-renewal and differentiation into mesenchymal cell lines, such as bone, cartilage, adipose tissue, neurons, or muscles.¹⁶ Typical MSC phenotypes include bone marrow-derived MSCs (BMSCs), tonsil-tissue-derived MSCs (TMSCs) and adipose-tissue-derived stem cells (ADSCs).

3D culture matrices provide oxygen and nutrients to stem cells, impact the alignment of the cytoskeleton and affect cell morphology, gene expression and protein production.¹⁷ In the past 10 years, extensive research has been devoted to understanding the factors that regulate MSC differentiation into



Scheme 9.1 Hydrogel-based 3D culture scaffold for stem cell culturing and differentiation.

specific cells and tissues (Scheme 9.1). The chemical functional groups, hardness or topography of the substrate, the size of the niche, and the geometry of the patterned surface can affect stem cell differentiation. The Jeong group¹⁰ have developed an injectable poly(ethylene glycol)-*b*-poly(L-alanine) (PEG-L-PA)s block copolymer that can be used to culture ADSCs. These PEG-L-PAs were composed of PEG with molecular weight of 5000 Da and L-PA with molecular weight of 620 Da, 1100 Da, or 2480 Da (Figure 9.4a). ADSCs could maintain spherical geometry within this hydrogel.

The effects of biomarkers lipoprotein lipase (LPL), type III β -tubulin (β Tub III), osteocalcin (OCN) type II collagen (Col II), and myogenic differentiation factor 1 (MyoD1) were studied for stem cell differentiation into fat, neuronal, bone and other tissue cells. *In vitro* studies of cells embedded in PEG-L-PA hydrogel indicated high expression of Col II and moderate expression of β Tub III and MyoD1 (Figure 9.4b), potentially developing ADSCs into chondrocytes. *In vivo* studies also showed that ADSCs could undergo chondrogenic differentiation (Figure 9.4c). However, biomarkers for lipogenesis (LPL) and osteogenesis (OCN) were both undetectable *in vitro* and *in vivo*.

In another study, Jeong's group developed poly(ethylene glycol)-poly(L-alanine-co-L-phenyl alanine) (PEG-PAF) thermogels for 3D TMSC culturing.¹⁸ This thermogel could serve as a support to encapsulate TMSCs together with various growth factors. The *in vitro* and *in vivo* results both indicated that type II collagen and sulfated glycosaminoglycan were highly expressed in TMSCs incorporated into PEG-PAF thermogel, indicating the preferential differentiation of TMSCs towards chondrogenesis. In this context, it can be concluded that cytoskeleton-matrix adhesion is an important trigger for stem cell differentiation.

As an improvement to a previous design, the same group also demonstrated a thermogel made of PEG-L-PAs that incorporated polystyrene

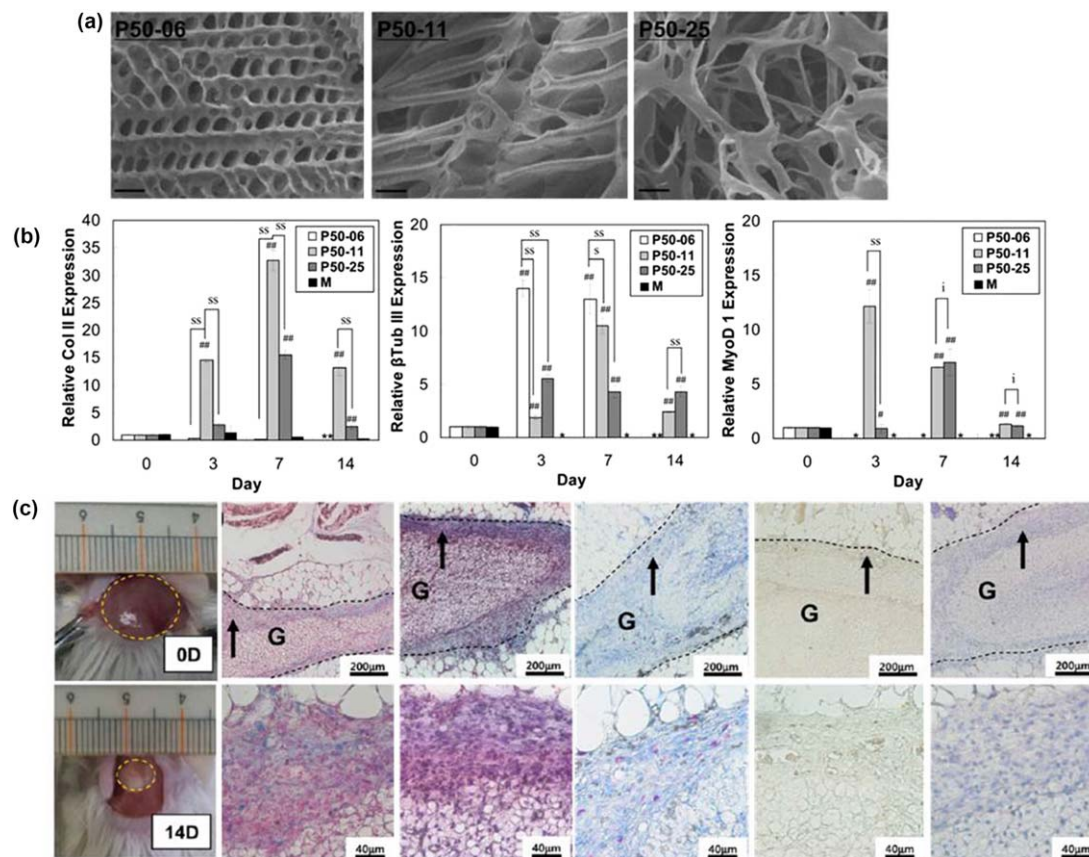


Figure 9.4 (a) SEM images of three kinds of PEG-L-PA thermogels prepared at 37 °C. The scale bar is 10 μ m. (b) *In vitro* studies, Col II, β Tub III, and MyoD1 gene expressions of ADSCs in PEG-L-PA thermogels. (c) *In vivo* mouse model of injected thermogel *in situ* with histological staining by alcian blue, Masson's trichrome, toluidine blue, alizarin red S, and oil red O around the implant at 14 days. The regions marked by arrows are enlarged. The thermogel regions (G) are specified by the dotted curve. The scale bars are 200 and 40 μ m, respectively. Reprinted with permission from ref. 10. Copyright 2013 American Chemical Society.

microspheres.¹⁹ These microspheres were designed to be within the size range 100 to 800 μm with various functional groups. An mRNA expression experiment indicated that the TMSCs differentiated into adipocytes in the thermogels with ammonium- or thiol-modified microspheres; differentiated into chondrocytes in a thermogel with thiol-, phosphate-, or carboxylate-modified microspheres; and differentiated into osteoblasts in the thermogels with phosphate- or carboxylate-modified microspheres (Figure 9.5a and b). TMSCs could be manipulated to preferentially differentiate into a specific cell type by controlling the functional groups of the microspheres in the thermogel.

9.3 Hydrogel-based 3D Scaffolds Induce Stem-cell-specific Differentiation

Chemical groups, stiffness, topography of substrates and geometry of surfaces can be manipulated to direct stem cell differentiation.^{20–22}

9.3.1 Scaffold-induced Neuronal Differentiation

Neurodegenerative injury or disease poses a serious threat to quality of life as we grow older and the ability of neural tissue for self-repair is limited.^{23,24} A recent study²⁵ suggests that PEG-L-PA thermogel might facilitate TMSC neuroforming (Figure 9.6a). In this study, microspheres, with loading of neuronal growth factors (*i.e.* BDNF or NGF, shown in Figure 9.6b), were co-encapsulated at a temperature of 37 °C. These cells showed exuberant vitality in this 3D culture medium and grew a structure that was similar to nerve fibers after a period of cultivation (Figure 9.6c). The associated neurobio-markers were highly expressed, suggesting that the cells could differentiate and form mature nerve cells.

9.3.2 Scaffold-induced Hepatogenic Differentiation

Liver disease is a leading cause of morbidity and mortality. Currently, the only effective treatment for acute liver failure and advanced liver disease is orthotopic liver transplantation.^{26,27} Donor organ deficiencies, surgical complications, immune rejection and expense limit this treatment option. Cell transplantation is a potential alternative.^{28,29} However, developing cell-based therapies for the treatment of liver diseases requires reliable and renewable sources of hepatocytes.

A PEG-L-PA thermogel system has been developed for growing hepatocytes.³⁰ Importantly, the sol-to-gel in this system undergoes transition at 37 °C to provide a gel with a modulus of about 1000 Pa, which is similar to that of acellular liver tissue. Hepatogenic growth factors present in the thermogel system change the morphology and aggregation of cells with expressions of hepatocyte-specific biomarkers and concomitant change of

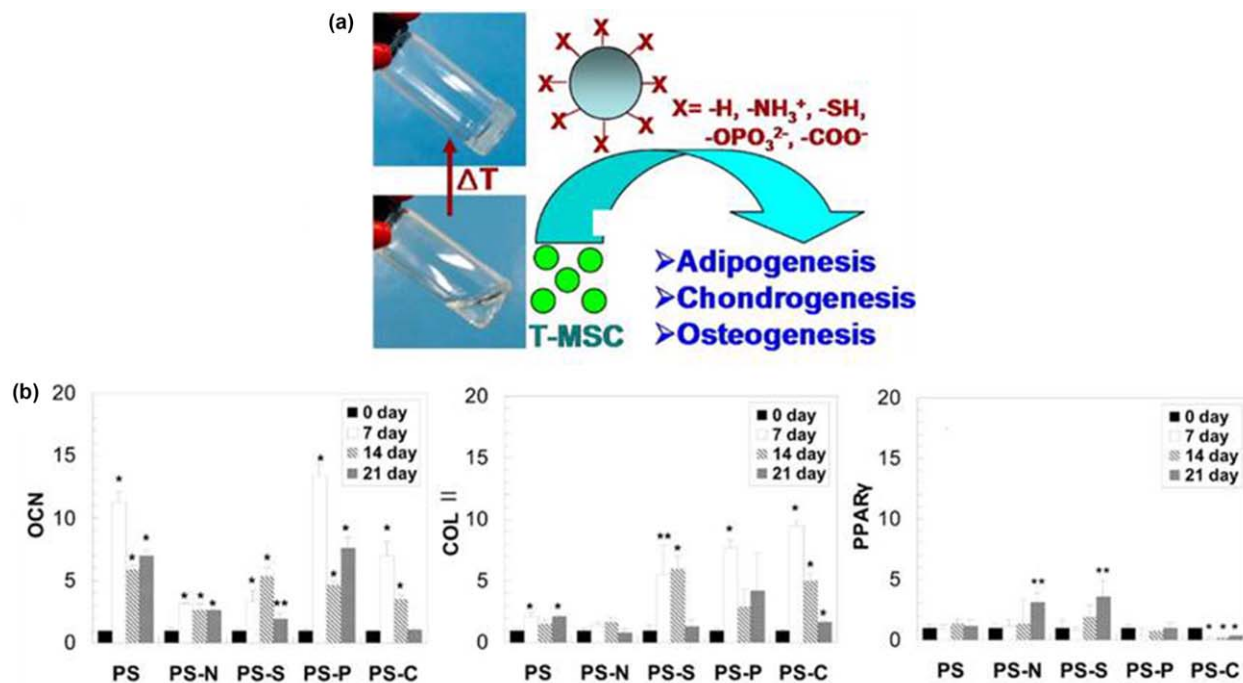


Figure 9.5 (a) Schematic of controlling specific differentiation of T-MSCs by adding surface-modified microspheres in PEG-L-PA thermogels. (b) OCN, Col II, PPAR γ expression of T-MSCs were analysis in 3D cultured thermogel. Reprinted with permission from ref. 19. Copyright 2014 American Chemical Society.

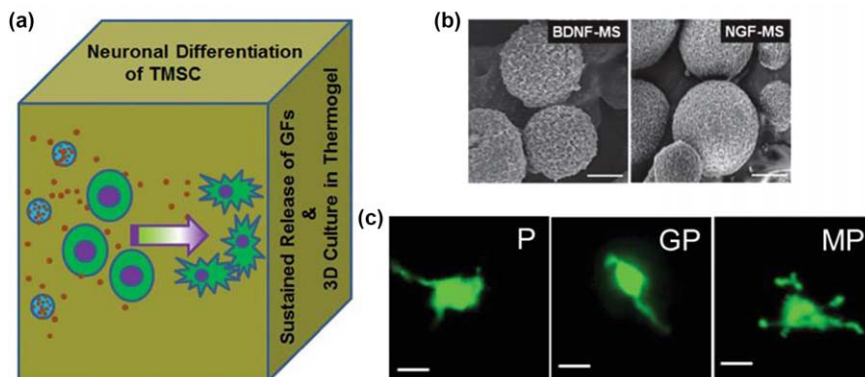


Figure 9.6 (a) PEG-L-PA thermogel as a 3D cell culture matrix incorporating TMSCs and neuronal growth factor loaded microspheres. (b) NGF-loaded and BDNF-loaded alginate microspheres. Scale bar is 1 μm . (c) Image of cells cultured in PEG-L-PA thermogel with different growth factors: P indicates the absence of growth factor, GP indicates thermogel incorporating growth factors, MP indicates thermogel incorporating growth factor-loaded microspheres. Scale bar is 20 μm .

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behavior of metabolic function. In the absence of hepatogenic growth factors, the production of hepatocyte biomarkers was slight and metabolic functions were weak. Production of albumin and α -fetoprotein were significant (Figure 9.7a) in both MGF (medium with hepatogenic growth factors) and GGF (3D matrix coencapsulated hepatogenic growth factors). The uptake of low-density lipoprotein (LDL) and cardiogreen (CG) was apparent in MGF and GGF (Figure 9.7b), which is typical metabolic function of hepatocytes. The conclusion can be drawn that the PEG-L-PA/TMSCs/growth factor system is a promising 3D scaffold for the differentiation of TMSCs into hepatocytes.

The Jeong group³¹ further investigated a PEG-L-PA thermogel system integrating TMSCs and hepatogenic differentiating factors. Hepatic biomarkers were expressed at both mRNA and protein levels and hepatic functionality such as CG and LDL uptake and albumin and urea production was observed (Figure 9.8).

9.3.3 Scaffold Induced Chondrogenesis Differentiation

Excellent viability was observed when BMSCs were encapsulated into both PEG-PA thermogel and Matrigel™ (Figure 9.9a). These BMSCs underwent chondrogenesis and myogenesis in the PEG-PA thermogel (Figure 9.9b), whereas neurogenesis was observed in the Matrigel™. In addition, an *in vivo* study in mice showed the prime formation of Col II and sulfated glycosaminoglycan (Figure 9.9c), indicating that chondrogenic differentiation of the BMSCs dominated in the implanted PEG-PA gel.³²

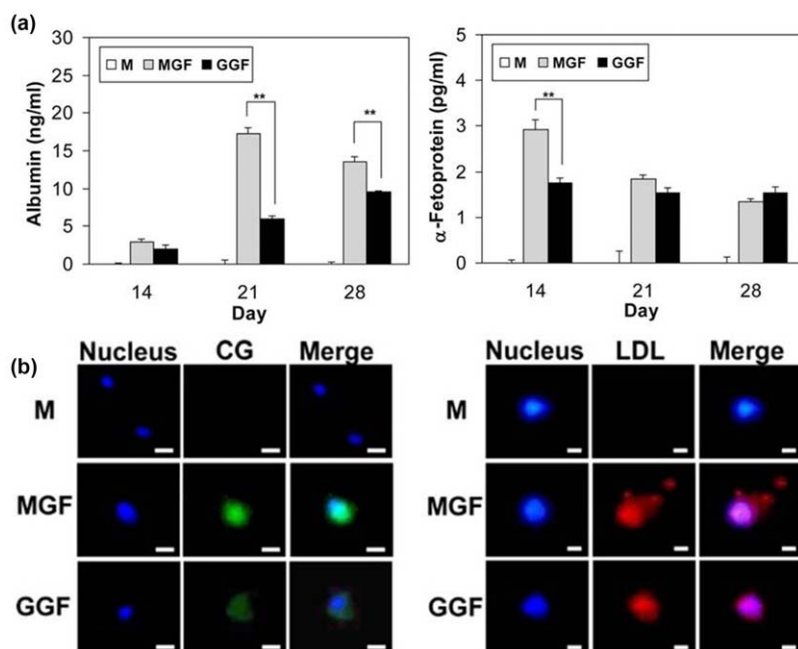


Figure 9.7 (a) Expression of albumin and α -fetoprotein by TMSCs in PEG-L-PA thermogel. (b) Images of cellular uptake of CG and LDL in PEG-L-PA thermogels at 28 days. The scale bar is 10 μ m. Reprinted with permission from ref. 30. Copyright 2014 American Chemical Society.

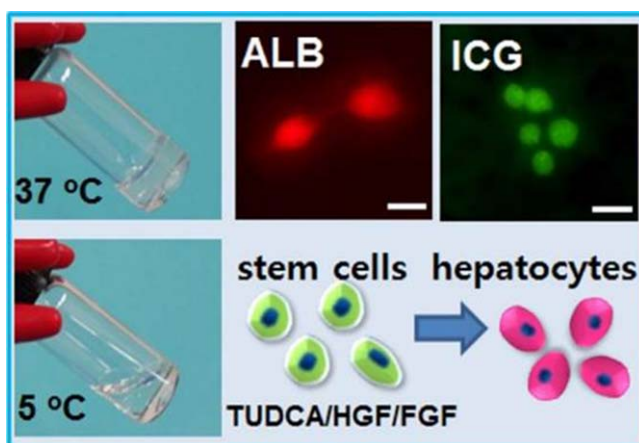


Figure 9.8 PEG-L-PA polypeptide thermogel system for hepatogenic differentiation of TMSCs. Reprinted with permission from ref. 31. Copyright 2017 American Chemical Society.

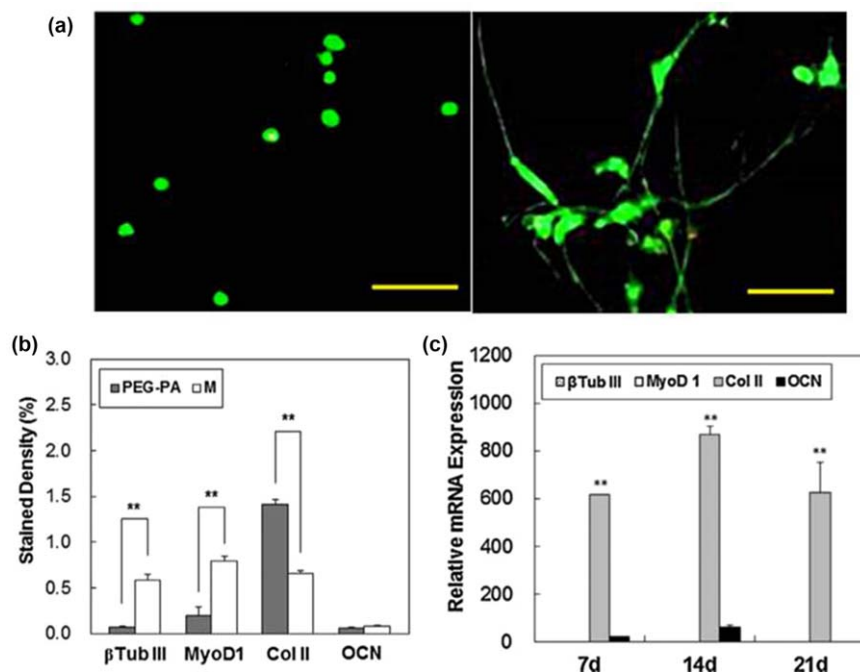


Figure 9.9 (a) Morphology of cells in the PEG-PA thermogel (left) and Matrigel™ (right) at the 21st day. The scale bar is 40 μ m. (b) Immunofluorescent assay using Image J software. (c) Gene expression *in vivo* of the BMSCs. Reproduced from ref. 32 with permission from John Wiley and Sons, © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Physicochemical parameters of structure such as topography, roughness of substrates, mechanical properties, and biochemical functional groups control stem cell differentiation. However, the dimensionality of the material, *i.e.* 1D fibers, 2D sheets, and 3D hydrogels, also plays a role. The Jeong group have developed a 2D/3D hybrid cell culture scaffold involving TMSCs suspended in thermogelling PEG-L-PA aqueous solution together with graphene oxide (GO) (Figure 9.10a).³³ The spherical morphology of the cells was maintained in this 2D/3D culture scaffold of GO/PEG-L-PA. Cells extensively aggregated when TGF- β 3-enriched chondrogenic culture media was used (Figure 9.10c), and expression of COL II, a chondrogenic biomarker, increased significantly (Figure 9.10b).

9.3.4 Scaffold-induced Osteogenic Differentiation

Every year there are a large number of clinical bone transplants, and with the aging population, osteoporosis is becoming an increasingly serious disease.³⁴ Osteogenesis through stem cell differentiation is considered to be a promising alternative to bone transplantation.^{35,36} Mesoscience is a key platform at the interface between biology and chemistry and encompasses

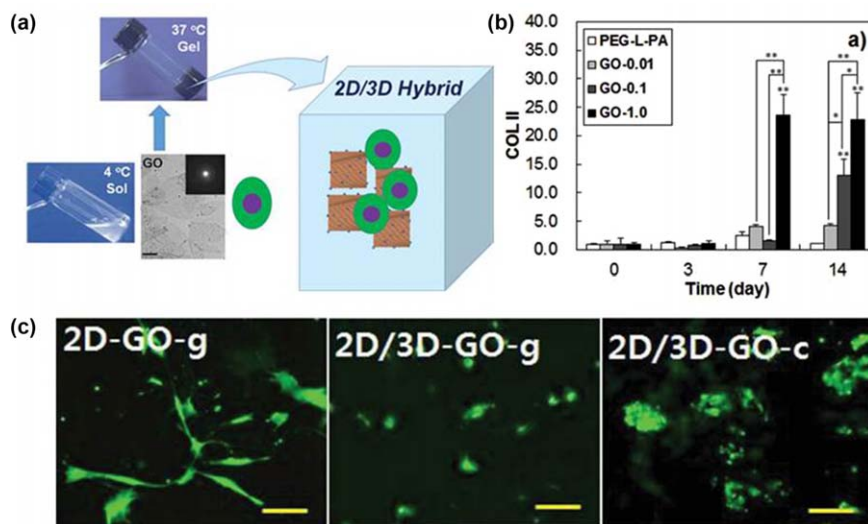


Figure 9.10 (a) Schematic of the 2D/3D thermogel system for culturing TMSCs. (b) Expression of COL II at different GO concentrations. (c) Cell morphology after 14 days of cell culture. Left: 2D culture with GO, middle: 3D culture with GO/PEG-L-PA, and right: 3D culture with GO/PEG-L-PA and enriched with TGF- β 3. The scale bar is 100 μ m. Reproduced from ref. 33 with permission from John Wiley and Sons, © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

the synthesis of precise mesostructures and characteristics for the cell-material interface. It is difficult for hydrogels themselves to function as a scaffold for osteogenesis due to the lack of a force transduction mechanism between the thermogel and cell. However, inorganic/organic mesocomposite materials can overcome this limitation of a single thermogel system for osteogenic differentiation. This technology involves mesocrystals embedded in the thermogel matrix to improve interactions between the cell and material leading to osteogenesis. One such system involves inorganic/organic complexes containing 4–8 μ m calcium phosphate mesogens (Figure 9.11a) which proved more effective at osteogenic differentiation of TMSCs relative to nanoparticle incorporating systems or hydrogels alone.³⁷ Osteogenic biomarkers (Figure 9.11c) at the mRNA and protein levels were highly expressed. This composite system provides both a hard surface for binding cells/proteins and a mild scaffold for holding cells in place (Figure 9.11b).

9.3.5 Scaffold-induced Adipogenic Differentiation

Adipogenic differentiation in a 3D culture scaffold can be used for reconstructive surgery of fat tissues.³⁸ Again, adipogenic differentiation was achieved in a 2D/3D culture scaffold by using TMSCs (Figure 9.12a). A graphene oxide/polypeptide thermogel (GO/P) system produced

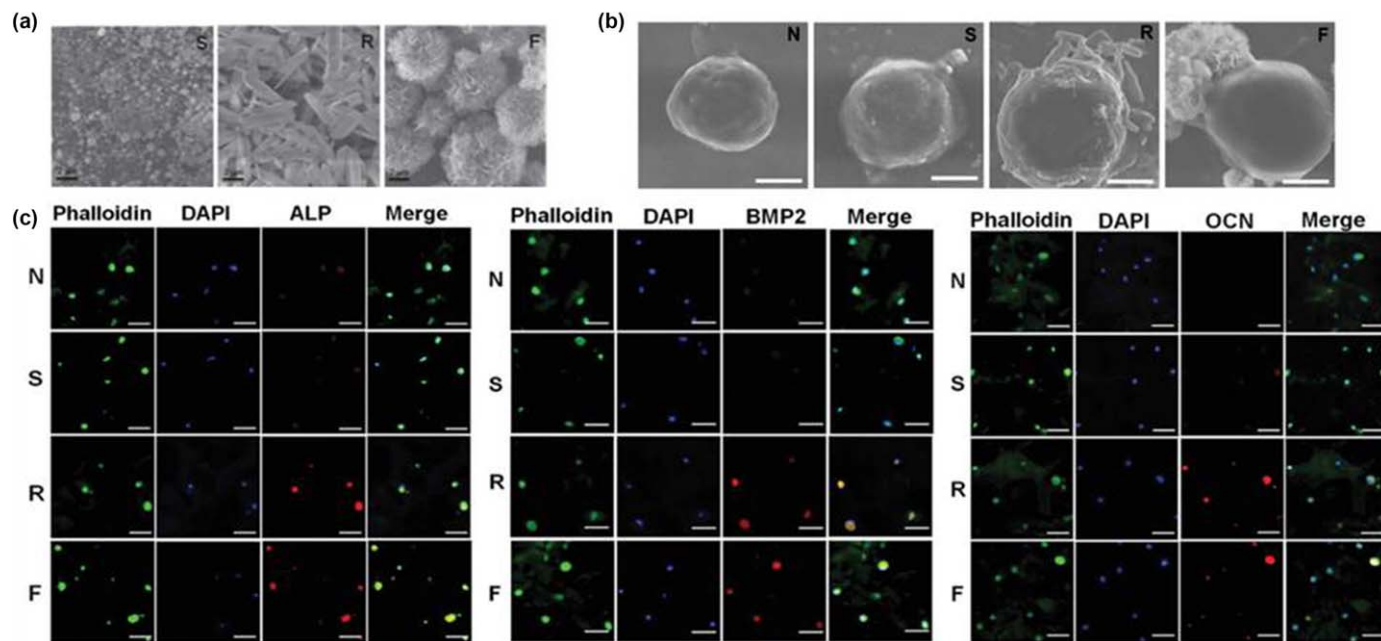


Figure 9.11 (a) SEM images of calcium phosphate crystals produced under different conditions. The scale bar is 2 μm . (b) SEM images of interactions between cells and cell culture matrix of thermogels. The scale bar is 5 μm . (c) Osteogenic biomarker production at protein level during the osteogenic differentiation of TMSCs. N represents the unmixed PEG-PAF. S represents the thermogel with calcium phosphate nanoparticles from Sigma-Aldrich. R represents the thermogel rod-like morphology calcium phosphate mesocrystals. F represents the thermogel flower-like morphology calcium phosphate mesocrystals. Reproduced from ref. 37 with permission from John Wiley and Sons, © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

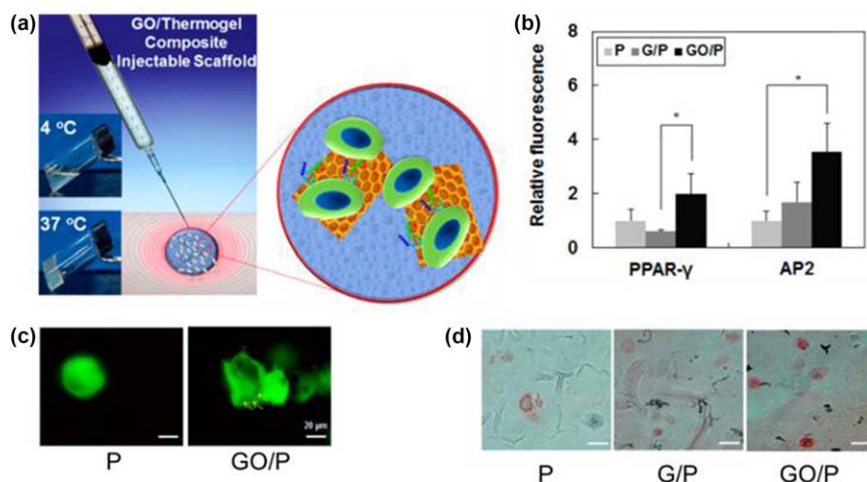


Figure 9.12 (a) Schematic presentation of the 3D culture of TMSCs in a GO/P composite injectable system. (b) Semiquantitative analysis of the fluorescence images. (c) Comparative images of a thermogel (P) system, and graphene oxide-incorporated thermogel (GO/P) system at day 14. The arrows in the GO/P system image indicate lipid vesicles. The scale bar is 20 μm . (d) Oil-red staining of the TMSCs incubating in the P, G/P, and GO/P systems, respectively, on the 14th day. The scale bar is 50 μm . Reprinted with permission from ref. 38. Copyright 2016 American Chemical Society.

adipogenic biomarkers, such as PPAR- γ , AP2 at a significantly enhanced level compared to a pure thermogel system (Figure 9.12b). Moreover, when insulin, an adipogenic differentiation factor, was added in the GO/P thermogel, it preferentially adhered to GO to provide sustained differentiation (Figure 9.12c). However, in the presence of G, insulin denatured partially and obstructed adipogenic differentiation (Figure 9.12d).

9.4 Conclusion

Adjusting the physical and chemical properties of a commonly used thermogel system provides a platform to manipulate stem cell differentiation by tuning substrate stiffness, porosity and ligand tethering. However, for practical applications of this scaffold in reconstructive surgery, efficiency of differentiation to adipose, hepatic and cartilage tissue needs to be improved. Thermogel composite systems are proving a promising tool, providing a minimally invasive injectable system, generating tissue volume and an excellent 3D matrix for differentiation of the incorporated stem cells.^{39,40}

Although 3D culture systems offer possible advantages relative to 2D culture systems, there are many constraints to be considered. Angiogenesis in these artificial 3D scaffolds, for example, is necessary to provide nutrients and remove waste.⁴¹ Moreover, it is challenging to alter one property of a

thermogel without disturbing other properties. Increasing stiffness, for example, will affect exchange of oxygen and nutrients in the thermogel. Effective control of stem cells will require scaffolds with multiple optimized properties including stiffness, biotic factors, biodegradability and porosity. Although a number of highly engineered, complex scaffolds have been designed over the past few years, most of these biomaterials are not yet suitable for commercialization.

On the positive side, modern microscale technologies open new opportunities. Scaffolds can be prepared using 3D printing and electrospinning at microscale, and even nanoscale, sizes.^{41–43} Spatial positioning of biomolecules and terrain of scaffolds can be accurately controlled. 3D thermogel scaffolds that control the growth and differentiation of cells will undoubtedly have a bright future in regenerative medicine.

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CHAPTER 10

Beyond Thermogels – Other Forms of Noncovalently Formed Polymeric Hydrogels

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10.1 Introduction

Hydrogels have a high water content, mild processing conditions, and are able to control the release of large macromolecules.^{1,2} Strong interest in hydrogels has developed due to their biocompatibility properties, and it is also possible to modulate the mechanical strength of hydrogels to match biological tissues.^{3,4} Covalently crosslinked chemical hydrogels have been formulated using techniques such as by inducing crosslinking using UV light or slower *in situ* crosslinking over time.^{1,5,6} The main issue with these methods is that the covalent networks formed are more permanent. It is more difficult to induce huge changes in hydrogel properties within an unchanging covalent framework. Therefore, to allow for dynamic changes in the hydrogel, for example for cell spreading and migration, polymer

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degradation is often necessary.⁷ In contrast, noncovalently formed hydrogels show reversible crosslinking with a kinetic rate that can change with the environment. Bonds which are broken due to injection or stress can be self-healed to reform the hydrogel. The hydrogel itself can also dynamically change over time without permanent changes to the hydrogel network.^{8,9} These noncovalently formed hydrogels have garnered strong interest recently due to these distinct and attractive properties, and there have been intense research efforts to better understand the benefits and applications of these hydrogels.

In this perspective, we discuss the main properties of noncovalently formed polymeric hydrogels, and cover recent developments in a few main classes of these noncovalent hydrogels. We highlight some common themes that run through the different noncovalent hydrogels, and provide an outlook for the potential as well challenges of the field. The topics chosen indicate some promising trends that we have seen and are not a comprehensive review of the entire field of noncovalently formed polymeric hydrogels. The reader is referred to several reviews that have a different angle and more detailed summary on related topics, whether focusing on supramolecular hydrogels, self-assembling hydrogels, thermogels or self-healing hydrogels.^{8,10–15}

10.2 Key Features of Noncovalent Polymeric Hydrogels

Noncovalent polymeric hydrogels are built up by reversible noncovalent interactions that are often constantly in a dynamic state of breaking and reforming or in a poised state for breaking once the correct stimuli is applied.^{16,17} Therefore, they exhibit several interesting macroscale properties, as presented in Figure 10.1.

- (i) Reversible/dynamic sol-to-gel transition: The non-covalent interactions are able to reform after breaking, and therefore these hydrogels are generally able to show reversibility. While there is a small degree of hysteresis occurring, the reformed hydrogels are able to recapitulate most of the mechanical strength and other properties of the original gel.¹⁸ They show various properties, including thermoreversibility when the temperature is changed, and mechanical reversibility when shear stress is removed, and they can also retain their original shape.^{19–21} The hydrogels are also highly dynamic, able to sample different states without permanent deformation and repair cracks and fractures, and they can allow for changes to facilitate cell migration/spreading.^{9,13,22}
- (ii) Tunable strength of molecular association: For regular covalent crosslinking, each individual crosslink shows a binary response of either bond formation or bond breakage, and the network strength is

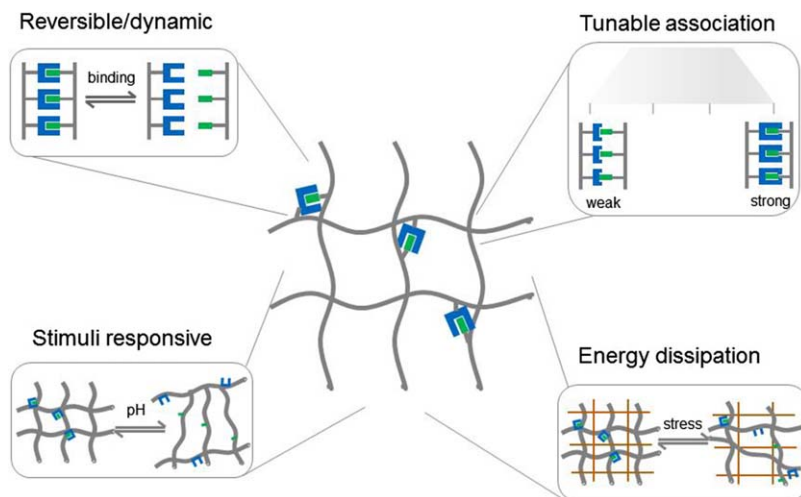


Figure 10.1 Non-covalent hydrogels show underlying properties of reversibility and tunable association, and this translates into macroscopic properties of stimuli responsiveness, tunable mechanical strength and energy dissipation.

tuned by number of crosslinks. For noncovalent hydrogels, the degree to which a noncovalent interaction is associated is determined by its concentration, c , and its kinetic exchange equilibrium constant.⁸ The equilibrium constant of each interaction, and consequently the bond strength, can be tuned by altering the pK_a/pH , changing neighbouring groups and switching around the noncovalent pairs,^{23–25} independently of the crosslinking density. Host–guest interactions can have equilibrium binding constants ranging from 10^3 to 10^{14} depending on the host guest pair, while metal ion interactions can exhibit equilibrium stability constants up to 10^{38} for the Tris–catechol– Fe^{3+} complexes.^{26,27} The interchange and lifetime of a complex can have a huge impact on material properties. Hydrogels with very slow exchange kinetics can show similar material properties as covalent hydrogels, while faster exchange rates can lead to characteristics of a viscous liquid. In addition, while changing the kinetic exchange rate can vary the dynamic properties greatly, the static properties, for example the Young's modulus, can remain quite similar.²⁵

- (iii) **Stimuli-responsiveness:** As the bonds are highly tunable, the bonds themselves can show variable responsiveness. The interchange and lifetime, as well as strength, can be changed with different environmental stimuli to create stimuli-responsive materials. Noncovalent hydrogels can be responsive to pH and ionic strength due to underlying electrostatic interactions, to redox based on thiol–disulfide exchange, to temperature due to the hydrophobic effect, to glucose due

to reversible boronate interactions between glucose and phenylboronic acid and to mechanical cues due to multiple weak non-covalent interactions that break upon high shear stress.^{10,17,23,28–30}

Furthermore, as responsiveness is mostly linked to some noncovalent interactions, these noncovalently formed hydrogels offer the potential of being maximally responsive materials. Noncovalent hydrogels expand the hydrogel response beyond swelling responses to stimuli-responsive degradation or dissociation while not necessarily being a destructive hydrogel response.³¹

- (iv) Energy dissipation: Noncovalent interactions have been frequently chosen as the sacrificial bond for forming tougher hydrogels. The bond can break under high stress to dissipate the strain energy, and it can at least partly reform again after the stress is removed to generate the original mechanical properties of the hydrogel.³² Interpenetrating hydrogel consisting of both covalent and ionic interactions have been used to form ultra-tough and stretchable hydrogels, and the utility of these hydrogels has recently been extended by showing that by including interacting groups on the surface, these hydrogels can also form strong adhesives. In addition to acting as one half of composite hydrogels, tough hydrogels have also been formed with purely non-covalent interactions *via* a combination of stronger and relatively weaker metal ion crosslinks.^{18,33,34} These hydrogels are highly stretchable, with the malleability to be stretched up to 20 times or more the original length, and the toughness to require very high fracture energies to break the material.

10.3 Types of Noncovalent Polymeric Hydrogels

Noncovalent polymeric hydrogels can be formed by crosslinking with various different types of noncovalent interactions, and the particular type of non-covalent interaction can affect the emergent hydrogel properties. We will be covering recent advances in the following main types of hydrogels: Supramolecular host–guest, hydrophobic association focusing on thermogels, ionic/metal ions and dynamic covalent.

10.3.1 Host–Guest-mediated Supramolecular Hydrogels

This section of the review discusses recent advances in supramolecular hydrogel assembly mediated by noncovalent macrocyclic host–guest interactions (Figure 10.2). The concept behind host–guest noncovalent interactions (electrostatic, Van der Waals forces, π effects, hydrophobic effects) to form supramolecular hydrogels relies on the reversible binding between complementary host–guest components.^{27,35,36} This section focuses on recent development of supramolecular hydrogels through macrocyclic host components' (e.g. cyclodextrins (CDs), cucurbit[n]urils (CBs), calix[n]arenes

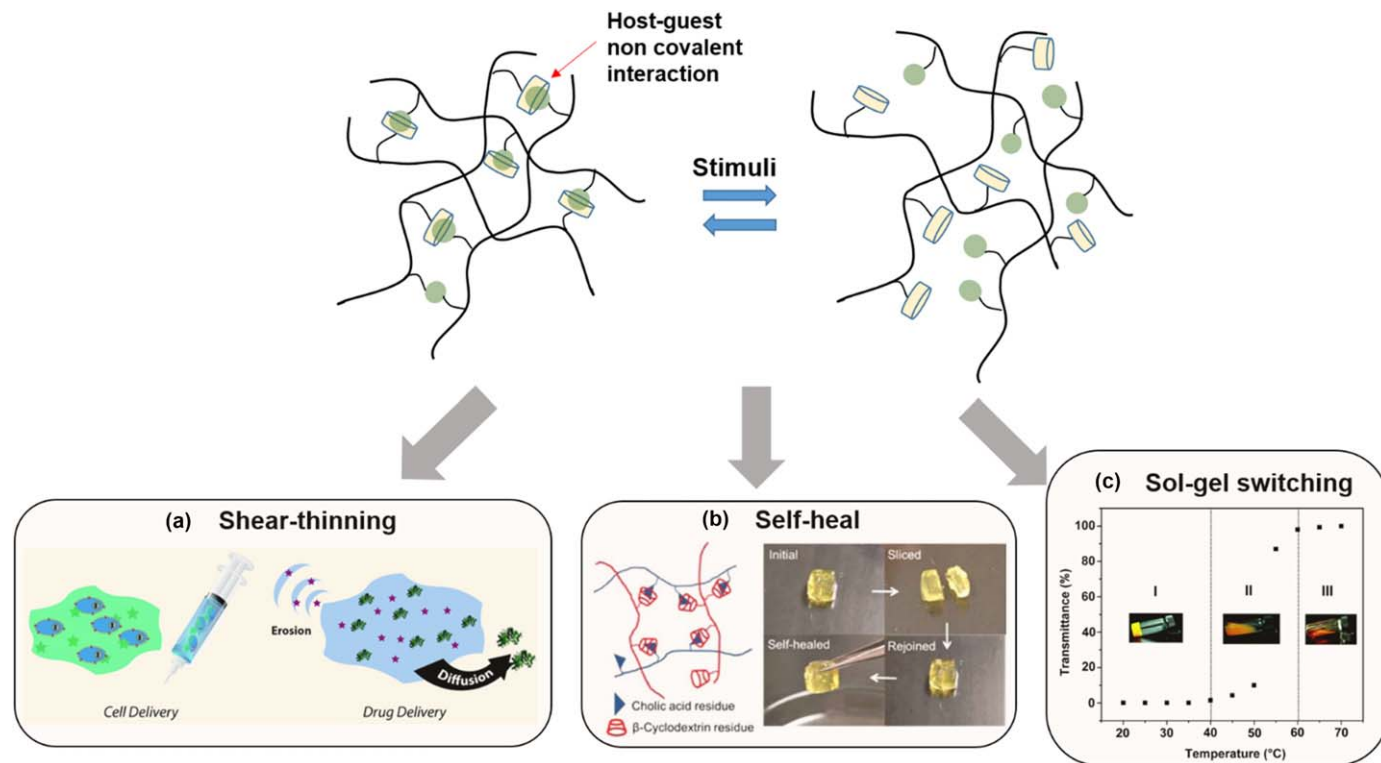


Figure 10.2 Schematic representation of supramolecular hydrogel network formation *via* host-guest interaction, exhibiting the following properties: (a) shear-thinning behavior for injectable cargo delivery (adapted from ref. 41 with permission from The Royal Society of Chemistry); (b) self-healing (adapted with permission from ref. 99. Copyright 2015 American Chemical Society); (c) sol-to-gel transition (adapted from ref. 53 with permission from The Royal Society of Chemistry).

(CNS), and pillar[*n*]arenes) hydrophobic internal cavity to accommodate different guest molecules.

The pioneering study by Harada to prove that poly(ethylene oxide) (PEO) could be threaded within multiple α -CD groups was an impetus to the research development of supramolecular assembled hydrogels.³⁷ Since then, a variety of polymeric architectures with host–guest interactions have been fabricated to form supramolecular hydrogel networks. Supramolecular hydrogels have been widely studied in the biomedical field as injectable material for therapeutic applications.^{38,39} Traditional covalent, crosslinked hydrogels required invasive surgical implantation to facilitate drug delivery. Supramolecular networks were developed to counter this by making the hydrogels injectable. Host–guest interaction is a notable strategy to fabricate reversible hydrogel. Due to the presence of noncovalent interactions within the network, studies demonstrated shear-thinning behavior of hydrogels to enable flow within a syringe and to self-heal into its reassembled form at the injection site.^{40,41}

Expanding upon this concept, hydrogels were designed by combining supramolecular and covalent crosslinking. Host–guest complexation within the hydrogel protected the secondary covalent network from bond rupture upon shear. For example, Burdick and co-workers designed methacrylated hyaluronic acid (Me-HA) supramolecular-double network hydrogel based on guest–host chemistry between β -cyclodextrin and adamantane and covalent crosslinking of MeHA.⁴² The application of this hybrid supramolecular-covalent hydrogel network was extended to bioink in 3D printing due to shear-thinning, self-healing, and tunable mechanical properties.⁴³ The study was expanded to include cationic polymer poly-ethylenimine (PEI) for compatibility to encapsulate nucleic acids for local delivery.⁴⁴

Our group studied the shear-thinning and self-healing properties of supramolecular hydrogels with different types of polymeric entities.^{45,46} Dynamic rheological studies confirmed that noncovalent inclusion complexation renders the sol-to-gel process reversible when subjected to shear and heating/cooling cycles. Our group also performed diffusion mechanism studies to demonstrate sustained release profile of these hydrogels to mimic real-time biological delivery.^{47,48} Building on the concept of reversible host–guest complexation, stimuli-responsive sol-to-gel switching materials were designed. Study of the light-induced healing of supramolecular polymer networks has gained momentum recently.^{49,50} Azobenzene-based hydrogels were the most studied owing to their visible light-induced response *via trans-to-cis* isomerization leading to sol-to-gel reversible phase of the hydrogel.⁵¹ Jiang and co-workers constructed a polypseudorotaxane (PPR) hydrogel between poly(ethylene glycol) (PEG) and α -CDs and the azobenzene derivative acted as a competitive guest that triggered *trans-cis* isomerization.⁵² Chen and co-workers recently developed a photo-responsive supramolecular hydrogel consisting of dimethylamino-substituted azobenzene entity (DAH) and α -CD. *Trans-to-cis* azobenzene transition triggered by light affected

host–guest interaction between azobenzene and α -CD, which induced sol-gel reversible phase.⁵³

Harada and co-workers looked at different systems to demonstrate the thermo-responsive association–dissociation of guest–host at different temperatures,⁵⁴ chemo-responsive competitive guest to trigger dissociation of host–guest,⁵⁵ pH-responsive protonation–deprotonation of guest molecules from host at different pH,⁵⁶ and redox-responsive guest molecule's dissociation from host cavity when oxidized⁵⁷ (25). With great strides in exploration of stimuli-responsive hydrogels being made, multifunctional supramolecular hydrogels with self-healing properties have become emerging areas of research.^{58–60} The design of such materials is valuable as it utilizes a simple molecular design that combines two or more types of noncovalent bonds that would enable the preparation of multifunctional supramolecular materials *via* polymeric design. Harada and co-workers designed a multifunctional coloring supramolecular hydrogel that could respond to chemical, thermal, and electric stimuli. In this study, they exploited the colour-changing property of phenolphthalein (PP) by preparing a β CD-PP AAm hydrogel based on acrylamide (AA) as the main chain and β CD and PP moieties as side chains. β CD-PP AAm hydrogel exhibits a colour change when heat or a competing molecule is applied at pH less than 8.⁶¹ The role of the supramolecular host–guest concept in hydrogel assembly has improved the functionality of materials. These hydrogels can respond to external stimuli, undergo self-assembly, sol-to-gel transition and self-heal. The current developments that exploit multiple responsive hydrogels provide the means to build more customizable structures, and these advances will lead to further critical development of improved supramolecular polymeric hydrogels.

10.3.2 Noncovalent Hydrogels through Hydrophobic Association

Polymers with hydrophobic and hydrophilic domains can self-assemble in aqueous solution, mainly driven by hydrophobic association. This phenomenon can also be used to form hydrogels with thermally reversible capabilities. Thermo-responsive hydrogels, also known as thermogels, exhibit reversible sol-to-gel transition as temperature changes.^{10,11,62} These physically crosslinked systems gain significant attention in biomedical applications such as drug release matrices for cancer or other treatment^{63,64} and cell culture scaffolds for tissue engineering,^{65,66} mainly because these systems possess advantageous: (1) they are injectable, so no open surgery is needed, and the hydrogel implant can form a gel at the desired location, while maintaining a liquid state in the needle; (2) they are degradable into small fragments that can be excreted by the body after serving their purpose, and the gel can be tailored to be degradable *via* hydrolysis, enzymatic degradation or triggered degradation;⁶³ (3) they are non-toxic, due to the high

water content in the hydrogel matrix; and (4) they are thermally reversible, so they can switch between sol and gel states without changing their original properties.

Thermo-responsive copolymers typically have amphiphilic structures that consist of hydrophobic and hydrophilic segments. These amphiphile structures self-assemble into polymeric micelles in water, with hydrophobic segments aggregating at the core of the micelles, and hydrophilic chains at the corona. Unlike covalent-crosslinked systems, the phase change behavior in the thermogel is reversible because the gel is formed *via* physical hydrophobic interaction. As such, optimizing the hydrophobic–hydrophilic balance in the thermogelling copolymer is the key to obtaining ideal thermogelling properties. Poly(ethylene glycol) (PEG) and poly(propylene glycol) (PPG) are widely used in thermogelling copolymers due to their well-known biocompatibility. In aqueous solution, PEG has a lower critical solution temperature (LCST) of 100–150 °C, while PPG has a LCST of 10–30 °C. Therefore, at low temperatures, both PEG and PPG domains are well dissolved in water; at body temperature, PEG is well-solvated in water, while PPG is becoming hydrophobic and not soluble in water. Taking the PF127 (poloxamer 407), for example, combination of PEG and PPG in a triblock structure (PEG₁₀₀-PPG₆₅-PEG₁₀₀), one can obtain effective thermogelation at physiological temperature. Besides PEG and PPG, typical polymers that show LCST include poly(*N*-isopropyl acrylamide) (PNIPAAm, 32 °C), poly(methyl vinyl ether) (PVE, 34 °C), and poly(*N,N*-diethyl acrylamide) (PDEAM, 25 °C). Refer to ref. 11, 67 and 68 for more examples of LCST polymers. PNIPAAm-based block copolymers were the reported in pioneer studies of thermo-responsive polymers for biomedical applications such as drug delivery, cell encapsulation, and the recent cell sheet engineering.⁶⁹ Typically, controlled RAFT or ATRP polymerization methods were employed to prepare these thermoresponsive block copolymer. Gupta *et al.* prepared PNIPAAm coupled with hydrophilic and soluble poly(*N,N*-dimethyl acrylamide) (PDMA) and hydrophobic block components of either poly(DL-lactic acid-*co*-glycolic acid) (PLGA), poly(ϵ -caprolactone) (PCL) or poly(propylene sulfide) (PPS).⁶³ In this study, drug release rates were found to be closely related with degradation of the hydrophobic blocks: PLGA (fast hydrolytic), PCL (slow hydrolytic), and PPS (reactive oxygen species (ROS)-degradable). In another study by our group, Barouti *et al.* designed the amphiphilic copolymer chain architecture (linear, triblock or star shape).⁷⁰ Poly(NIPAAm) block copolymers with linear chain structure showed fastest drug release rate compared with triblock and star-shaped copolymers because they contained more entanglement and bridging chains in their micellar networks.

In another aspect, the classic triblock A-B-A type thermogelling copolymer, which consists of PEG and PPG, is the most commonly studied structure. Recent reports on modification of PEG-PPG-PEG (PF127) end groups could improve the mechanical properties and enhanced gel stability against heat and water erosion.⁷¹ The group modified PF127 with diphenylalanine (FF) end groups to produce a stable gel up to 90 °C, and the gel could withstand

water erosion for 60 h (instead of 24 h for unmodified PF127 gel). In another study, poly(DL-lactic acid)-PEG-poly(DL-lactic acid) (PDLLA-PEG-PDLLA) triblock thermogelling copolymer was synthesized.⁷² The gel showed good properties as a physical barrier in rat model, enhanced tissue regeneration and degraded after 14 days. Besides, nanocomposite thermogel, poly(DL-lactic acid-co-glycolic acid)-PEG-poly(DL-lactic acid-co-glycolic acid) (PLGA-PEG-PLGA) blended with laponite clay, showed enhanced tissue regeneration and functional recovery.⁷³ Laponite in the gel is able to absorb host-derived bioactive molecules and retain them for the formation of native ECM after degradation of PLGA-PEG-PLGA hydrogel. For specific application in the eye, an injectable therapeutic matrix based on triblock (PLGA-PEG-PLGA) was successfully tested in an *in vivo* study for glaucoma treatment.⁶⁴ In this study, the thermogel showed sustained delivery of at least 7 days, and could effectively modulate the relief of intra-ocular pressure.

The formation of polyurethane is a relatively easy way of synthesizing thermoresponsive copolymers *via* a typical one-pot polycondensation reaction. Recent advancement in this area is mostly focusing on minimal invasive cancer therapy and bioimaging. Our group has been developing thermogelling polyurethanes based on PEG and PPG, with the third hydrophobic component being either poly(L-lactide) (PLLA),⁷⁴ poly([R]-hydroxybutyrate) (PHB),⁷⁵ or poly([R]-3-hydroxybutyrate-co-4-hydroxybutyrate) (P3HB4HB),⁷⁶ to impart biodegradability. Thermogels with PLLA and PHB were used as drug delivery depot for treating cancerous animal models.^{74,75} These drug-loaded thermogels could effectively inhibit the growth of cancer cells. Besides, thermogelling polyurethanes based on PEG and PPG were synthesized with a third component being a light-responsive moiety such as aggregation-induced emissive (AIE) molecules: tetraphenylethene (TPE)⁷⁷ and spiropyran.⁷⁸ Thermogels with TPE could be used as effective bioimaging probes. Unlike typical fluorescent molecules, which exhibit self-quenching due to aggregation in the body, thermogel with AIE properties showed exceptionally high emission from agglomeration of micelles, as shown in Figure 10.3. This is beneficial for the application of long-term tracking of drug molecules, as the fluorescent signal from the gel was detectable up to 18 days *in vivo*. On the other hand, a thermogelling copolymer that consisted of spiropyran showed fast reversible gelation due to both light and temperature. This thermogel may find application as a photo-responsive sensor or anti-counterfeiting material.⁷⁸ Hydrogels formed *via* hydrophobic interaction, shown using the example of thermogels, possess many advantageous properties suitable for biomedical applications. However, they also show some limitations, such as low tensile strength, that restrict their usage as load-bearing matrices, and fast gel erosion that could result in burst release of drugs. Along with proper modifications (*e.g.* blending with clay or end group modification, *etc.*), one can reduce the impact of these limitations. In addition, hydrogels formed *via* hydrophobic association can be exploited for more applications beyond biomedical applications, such as anti-counterfeiting materials, food texture modifiers, and skin care products.

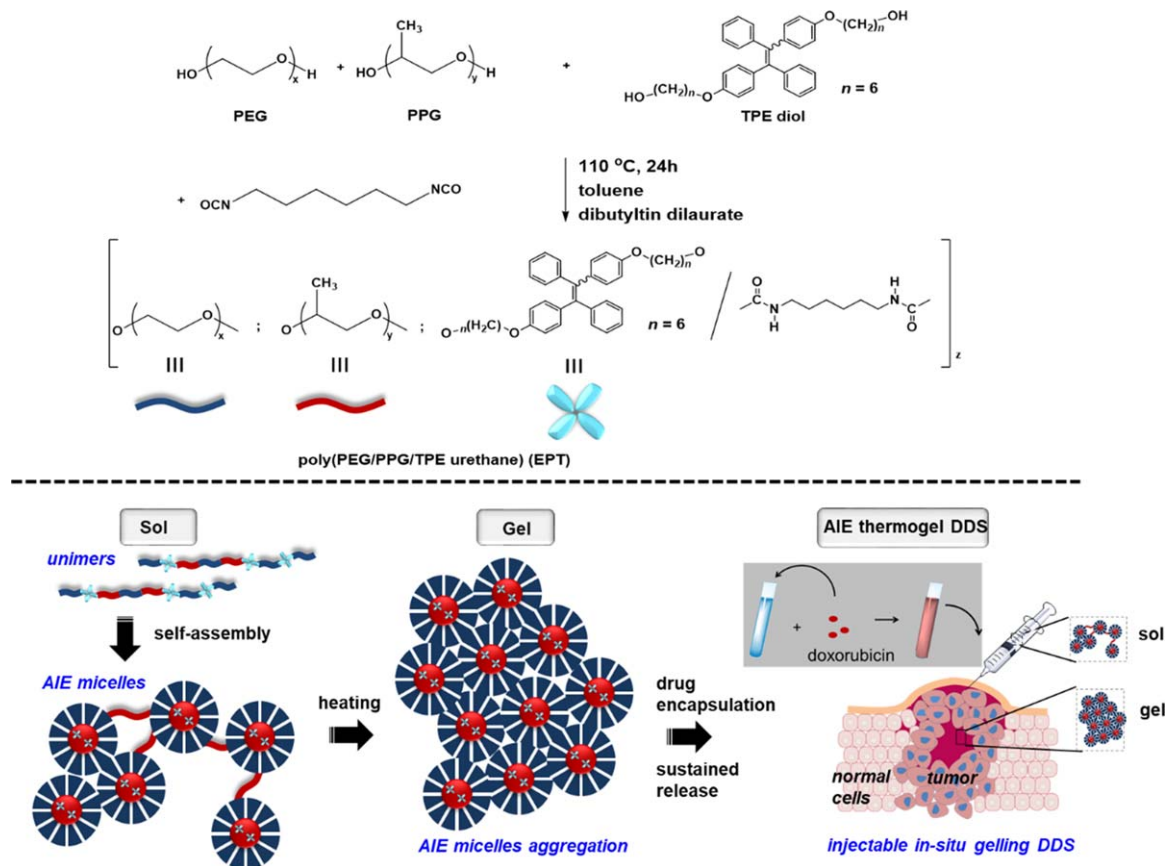


Figure 10.3 Synthesis procedure of EPT thermogelling copolymer, and schematic illustration showing the use of EPT thermogel as *in situ* gelling drug delivery carrier.
Adapted from ref. 77 with permission from John Wiley and Sons, © 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

10.3.3 Noncovalent Polymeric Hydrogels Through Forming Ionic Bonds

Ionic bonds refer to electrostatic interactions, for example between chitosan and alginate polymers. These interactions can range in strength from a few carboxyl groups on a long polymer chain, to regularly repeating carboxyls on a polyacrylate, to the higher affinity coordination bonds with metal ions. While these interactions are strong, there are often charge shielding effects under physiological salt concentrations. Therefore, the polymer with a repeating charge unit and the multidentate higher affinity metal ion coordination bonds are the main interactions used for building up non-covalent hydrogel systems.^{79,80}

As an example, the metal ion coordination systems are highly tunable without polymer modification required. Imidazole-containing polymers (ICPs) could be crosslinked by the different metal ions Co^{2+} , Zn^{2+} , or Cu^{2+} , and the imidazole ligand:metal ion ratio can also be varied to change the mechanical characteristics from viscous fluids to stiff plastics. When the ligand:metal ion ratio corresponded to the coordination number of the metal ion, the bulk material presented itself as a relatively stiff plastic. Interestingly, as the unbound imidazole was increased, the bulk material became an extensible rubber. When the ligand:zinc ratio was increased from 4.0 to 4.5, there was a decrease in tensile strength by 540%, but also an increase in extensibility by 600%. By including a small proportion of unbound ligand, the crosslink exchange rate increased, and the gel additionally showed properties of self-healing. Therefore, contrary to expectations, where only crosslinked molecules affect mechanical properties, the unbound ligands play an important role in stress distribution and chain relaxation.²⁴ Instead of replacement with a new metal ion–ligand pair to tune properties, it is also possible to have two metal–ligand crosslinks of different kinetic exchange rates concurrently present within the same material, and it was shown that the mechanical properties of the hydrogel could be modified by tuning the concentration of each metal ion–ligand pair relative to each other.⁸¹

It is possible to incorporate ionic charges to lead to charge-induced assembly and to also generate other desirable properties. Amphiphilic BAB triblock copolymers with a central PEG group can have B groups swapped out to charged polyglutamic acid and poly-L-lysine. Combining separate polymers with polyglutamic acid and poly-L-lysine now leads to charge-induced assembly of hydrogels, instead of regular hydrophobic-induced assembly. Relatively strong hydrogels with a storage modulus of around 10 kPa were formed with a lower molecular weight PEG of 1.5 kDa. These hydrogels could disassemble reversibly at a $\text{pH} < 3$ or > 11 .²⁸ Adding imidazolium bromide or zinc dimethacrylate to rubber allowed ionic crosslinks to form, and better mechanical properties were observed. These modifications also introduced self-healing properties, which could be useful in inducing rearrangements in rubber.^{82,83} Alginate is commonly used to form ionic gels

with divalent cations such as calcium, and the alginate polymer can be grafted onto hyaluronic acid (HA) to allow for gel formation in the presence of calcium ions instead of covalent crosslinkers.⁸⁴

Ionic bonds are also frequently used as sacrificial bonds to dissipate energy. Tough hydrogels can be formed from interpenetrating networks of covalently formed polyacrylamide and ionically formed alginate–calcium, and these hydrogels can be stretched up to 20 times their original length, and also have fracture energies of $\sim 9000 \text{ J m}^{-2}$.^{33,85,86} These tough hydrogels can be further functionalized with adhesive surfaces to form super strong adhesives.³⁴ By attaching the ligand 2,6-pyridinedicarboxamide, poly(dimethylsiloxane) polymer chains form both strong and weak metal ligand-binding interactions to Fe(III). This generates a highly stretchable material which can now be extended to 45 ± 2 times its original length and a fracture energy of around $2571 \pm 20 \text{ J m}^{-2}$. This is because the weaker carboxamido–iron bonds can be broken with stress and allow the polymer chains to progressively unfold and extend out, while the strong pyridyl–iron bonds ensure that the polymer is still attached to iron even under high stress (Figure 10.4). The material can also recover when the strain is removed, and self-heal at a low temperature of -20°C .¹⁸ It is alternately possible to create tough hydrogels by copolymerizing acrylamide and the negatively charged 2-acrylamido-2-methyl propane sulfonic acid together with aluminium hydroxide nanoparticles. These gels can be extended up to 21 times their original length and show a high compressive strength of 18.9 MPa .²¹

These ionic hydrogels have tunable stress relaxation properties which could be applicable to tissue engineering. It was found that cells spread to a larger degree on softer viscoelastic hydrogels with stress relaxation compared with elastic hydrogels of the same gel modulus.⁸⁷ This was investigated further by studying a series of alginate–calcium hydrogels with stress relaxation properties tuned mainly by varying the alginate polymer molecular weights from 280 kDa to 35 kDa, and the range of timescales achieved from $\sim 1 \text{ h}$ to $\sim 1 \text{ min}$ is similar to that of viscoelastic biological tissues. With faster stress relaxation, there is an increase in cell spreading and proliferation for fibroblasts, as well as an increase in osteogenic differentiation for mesenchymal stem cells.²² These viscoelastic noncovalent hydrogels might be interesting to study further for cell–ECM and tissue engineering, as most of the extracellular matrix in the body shows stress relaxation to some extent. Ionic linkages show high tunability depending on the charged group or metal and ligand chosen, and can also be introduced into existing systems to provide self-healing or pH responsive properties. Ionic linkages have also been frequently used as sacrificial bonds in combination with covalent bonds or stronger versions of itself. Metal ion–ligand interactions have been generally regarded as the strongest noncovalent interactions within physiological environments, and we expect that there will be lots of potential future developments harnessing this important characteristic.

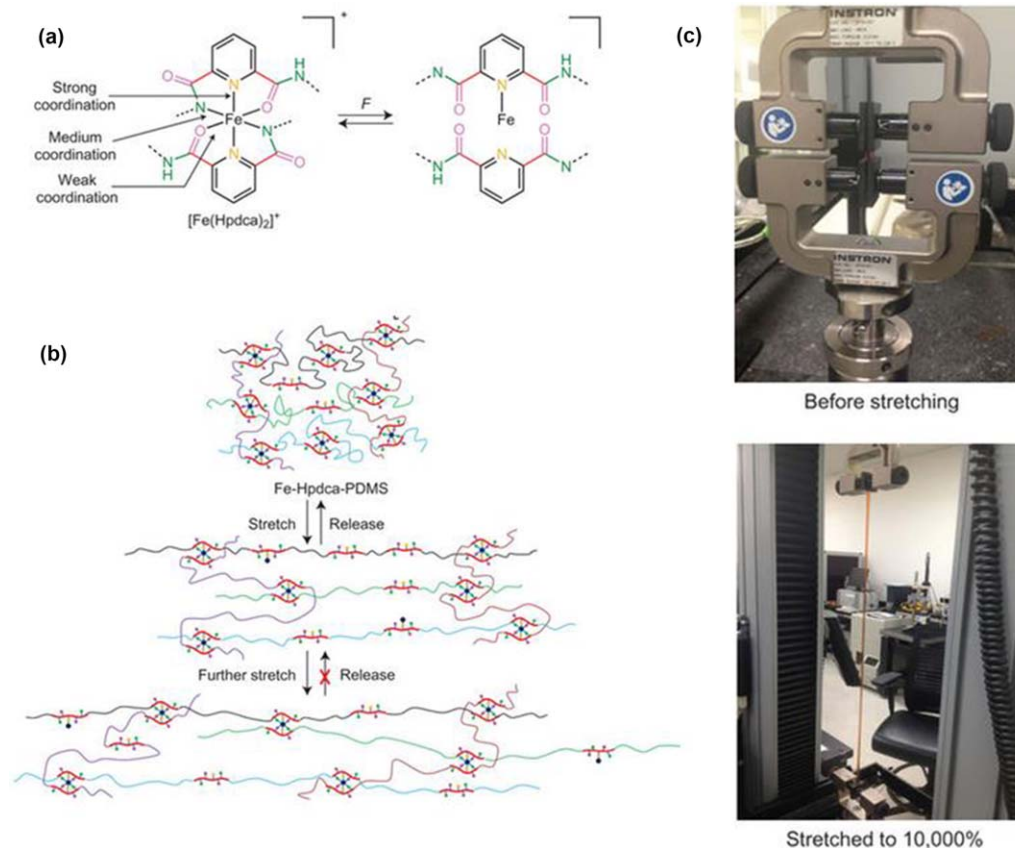


Figure 10.4 Combination of weak and strong metal–ligand interactions leads to highly stretchable polymer. (a) Structure of the $[\text{Fe}(\text{Hpdca})_2]^+$ moiety undergoing reversible rupture and reconstruction during tensile stretching (using a force F) of the films. (b) Structure of the material, and proposed mechanism for chain folding and sliding during tensile stretching. PDMS chains shown in different shades represent different polymers. (c) Photographs of a film (with an Fe(m) metal to H2pdca-PDMS ligand molar ratio of 1:6) before and after stretching to 10 000 $\times$. Adapted by permission from Macmillan Publishers Ltd: *Nature Chemistry* (ref. 18), copyright 2016.

10.3.4 Dynamic Covalent Bond-based Polymeric Hydrogels

Dynamic covalent bonds are not like the typical covalent bonds which are permanent and irreversible. They are a unique niche group that possess some covalent bond characteristics, but also exhibit bonds which can repeatedly attach and disengage. In comparison to most of the noncovalent interactions described earlier, dynamic covalent bonds have a higher strength per bond interaction.⁸⁸ They can be as stable as covalent bonds in some cases, but can also be induced to rapidly exchange, mostly when a stimuli or catalyst is present.⁸⁹ There is a small but expanding group of a few different dynamic covalent bonds which have been applied to hydrogels. These are principally the boronate esters and disulfides, as well as imines/hydrazones.¹⁶ By changing the neighbouring groups, it is possible to tune the exchange kinetics of the boronic ester. While regular phenylboronic ester (PBA) showed a transesterification rate of $0.016 \pm 0.004 \text{ s}^{-1}$, the dimethylaminomethyl phenylboronic acid showed faster rates of transesterification of 3000 s^{-1} . Gels formed from the regular PBA showed a higher storage than loss modulus with a crossover frequency of less than 0.01 Hz, behaving almost like a covalent hydrogel. Gels formed from the modified PBA show viscoelastic mechanical properties, a faster stress relaxation of 5 min and also showed self-healing properties (Figure 10.5). While there is a large difference in the dynamic properties, the static mechanical property of Young's modulus was much more similar, showing that these properties can be independently modulated.²⁵ When PBAs of different pK_a were tested, it was found that the PBA with the lowest pK_a showed the earliest crossover where storage modulus was higher than loss modulus, therefore leading to a more elastic mechanical behavior.²³ In contrast, for a single polymer chain composed of both PBA and glucose moieties, these gels showed storage modulus greater than loss modulus at all frequencies.⁹⁰ It is interesting that the phenylboronate ester shows properties varying from viscoelastic to elastic based on different studies, and it might be insightful if a kinetic study was conducted across the board to elucidate the key predictive factor for gel properties of these phenylboronate ester gels.

Hydrogels based on gold-thiolate/disulfide exchange allows for the properties of injectability and self-healing. They show frequency-dependent mechanical stiffness similar to that of other noncovalent hydrogels. Cyclic tension/compression tests show that they have similar biomechanical shock-absorbing properties to the nucleus pulposus in intervertebral discs, indicating that it has some potential application for replacement of shock-absorbing tissues.²⁹ Thiol-disulfide exchange has previously been used to allow hydrogel formation to be initiated and stopped by changing of the pH in the system in a “living” controlled hydrogel formation.⁹¹ This pH responsiveness has also been an important property of the dynamic hydrogel. Triblock copolymers with terminal dithiolane blocks can show reversible ring opening and crosslinking of the 1,2-dithiolanes and show self-healing properties. However, at lower pHs where this thiolane exchange does not

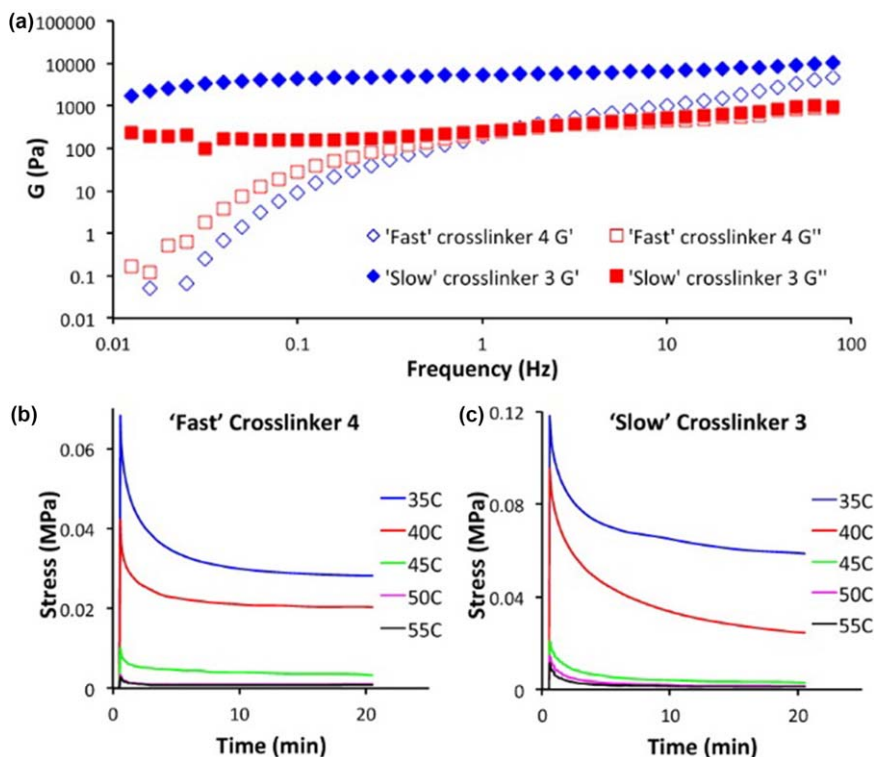


Figure 10.5 Speed of kinetic exchange affects whether the dynamic boronate gel shows rheological properties of viscoelastic noncovalent or elastic covalent bonded hydrogels. (a) Rheological data showing storage (diamond) and loss (square) moduli for samples cross-linked with fast modified PBA crosslinker compound 4 (unfilled) and slow regular PBA crosslinker compound 3 (filled). (b, c) Stress relaxation data for 20%-diol PCO cross-linked with diboronic esters 4 (b) or 3 (c). Adapted with permission from ref. 25. Copyright 2015 American Chemical Society.

occur, the gel is kinetically locked in place and loses its dynamic properties.⁹²

A hydrogel can be formed by two concurrent dynamic covalent bonds, the imine bonds between *N*-carboxyethyl chitosan (CEC) and oxidized sodium alginate (OSA), as well as the acylhydrazone bonds between adipic acid dihydrazide (ADH) and OSA. The storage modulus of the hydrogel can reach almost 6000 Pa. The hydrogel also shows a high healing efficiency of up to 95% when kept for 12 h.⁹³ PEG crosslinked with hydrazones were investigated for their dynamic degradation properties with multiple particle tracking microrheology. At pH 4.3, when the gels are pushed out of equilibrium, the critical exponent value shows a tightly crosslinked network, and the hydrogel degrades rapidly over several hours. At pH 7.1, the critical relaxation exponent value is 0.86 ± 0.04 , which represents a loosely crosslinked

network that loses energy. There is bond breakage and hysteresis in bond reformation, leading to the scaffold degrading slowly over 1.5 weeks. This study provides a better understanding of degradation of these hydrazine linkages.⁹⁴ It is also possible to tune the degradability of dynamic hydrogels from less than 24 h to more than 7 days by changing the proportion of the more stable oxime linkages and the less stable hydrazine linkage.^{95,96}

These dynamic covalent gels can also be applied to tissue engineering. Hydrazone linkages can be attached to PEG gels, and show a Young's modulus of 1.8–27 kPa. Hydrogels formed using acyl aldehydes showed fast stress relaxation and myoblasts were able to spread and extend out filopodia. In contrast, hydrogels formed with aryl aldehydes showed slower relaxation, and most cells were still rounded after 10 days.⁹⁷ An interpenetrating network hydrogel was formed from HA crosslinked with dynamic hydrazone bonds and collagen fibrils. The dynamically crosslinked HA hydrogels alone show that the storage modulus is greater than the loss modulus at all frequencies, thereby showing a stable covalent-like network. The combination hydrogel showed two different types of stress relaxation based on its components, and faster relaxation increased cell spreading as well as formation of focal adhesions. These hydrogels would be useful in recapitulating both the viscoelasticity and fibrillarity of the ECM.⁹⁸

10.4 Summary and Outlook

Noncovalent hydrogels have unique properties that are different from those of covalent hydrogels. These hydrogels show reversibility, including self-healing properties after removal of shear stress. Noncovalent hydrogels exhibit tunable mechanical strength, which can range from a viscous liquid to an elastic gel based on modulating the association exchange rates. Noncovalent hydrogels also show an ability to respond to and influence the environment, with gels that can dissipate mechanical energy and others that can respond to glucose, as well as inherent stress relaxation properties that allow for cell spreading and proliferation.

The non-binary bond strength provides a lot of tunability beyond what covalent hydrogels can offer, and there is still much more that can be realized about tunability and the potential applications of this powerful optimization tool. The dynamic covalent interactions have still been a relatively niche topic compared to the other noncovalent interactions, and there are other reversible reactions belonging to dynamic covalent chemistry which can be researched further and potentially be utilized to contribute to self-healing gels. As some of the examples show, the properties of these hydrogels can already span the spectrum between almost-covalent to viscous liquid based on the kinetic exchange rate. The dynamic properties allow these hydrogels to change in response to the environment, whether it be chemical signals or cellular signals for migration and movement. There have been exciting developments demonstrating that these hydrogels can show the optimum stress relaxation properties for cell spreading, and it is possible

that these hydrogels could mimic the changing physiological environmental much better than existing elastic hydrogels. With the intense research going into this field, it is highly likely that some other aspect of cellular or tissue function will be highly dependent on these hydrogels.

Many noncovalent gels have not yet found practical applications, mainly due to their poor mechanical properties. There is still a big gap between regular dynamic gels and tough gels that can sustain stress at the MPa level and exhibit fracture energy of the order of 1000 J m^{-2} . In the past few years, there has been remarkable progress in developing tough self-healing hydrogels, although these are still isolated circumstances and it will be imperative to understand the general mechanisms that allow self-healing gels to show tougher mechanical strength. It is also an open question about how these self-healing properties can be applied beyond simple self-healing studies. Can they actually repair cracks or heal gaps in a useful manner, and are there more situations where these weaker soft-healing gels can play a more suitable role than stronger covalent gels? This is a promising field, and over the coming years we expect many exciting developments that will serve to delineate the directions worth pushing forward into applications.

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